UNIVERSITY OF ALBERTA

Identification of Mitochondrial Specific Copper Chaperones and Functional Analysis of Human COX17 "pseudogene."

by

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ABSTRACT

Cox17p is essential for respiratory growth in yeast and is thought to deliver copper to Cox11p and Sco1p for subsequent delivery to cytochrome oxidase (COX). Human homologs of these proteins exist and are thought to function similarly to those in yeast. COX deficient patients have been found to bear mutations in *SCO1*, but not in *COX17* or *COX11*. In this thesis, I demonstrate by a yeast high-copy suppression screen that other gene-products must be involved in copper delivery to Sco1p. As well, I demonstrate that COX17P (product of human COX17 pseudogene) and COX17 localize to both the cytoplasm and mitochondria. COX17P may function in mitochondrial copper delivery and provide functional redundancy in the COX assembly pathway, which would explain why no *COX17* mutations have been found to cause human COX deficiencies. *COX17P* is expressed in several types of immune cells, which may suggest an immune-specific role for COX17P.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
AMP	ampicillin
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
COX	cytochrome oxidase
COX17P	COX17 pseudogene
Cu	copper
ddH_2O	deionized, distilled water
EG	ethanol/glycerol
ETC	electron transport chain
EST	expressed sequence tag
GABP	GA-binding protein
GAL	galactose
IMM	inner mitochondrial membrane
IMS	intermembrane space
kb	kilobases
kDa	kilodaltons
LB	Luria-Bertani
LS	Leigh syndrome
LSFC	Leigh syndrome, French Canadian
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtTFA	mitochondrial transcription factor A
NADH	nicotinamide adenine dinucleotide, reduced
nDNA	nuclear DNA
NMR	nuclear magnetic resonance
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PMS	post mitochondrial supernatant
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
UTR	untranslated region
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD1	Cu, Zn superoxide dismutase
TF	transcription factor
tRNA	transfer RNA
WO	minimal glucose media
YPD	yeast peptone dextrose (rich glucose medium)

Chapter 1 ❖ Literature Review

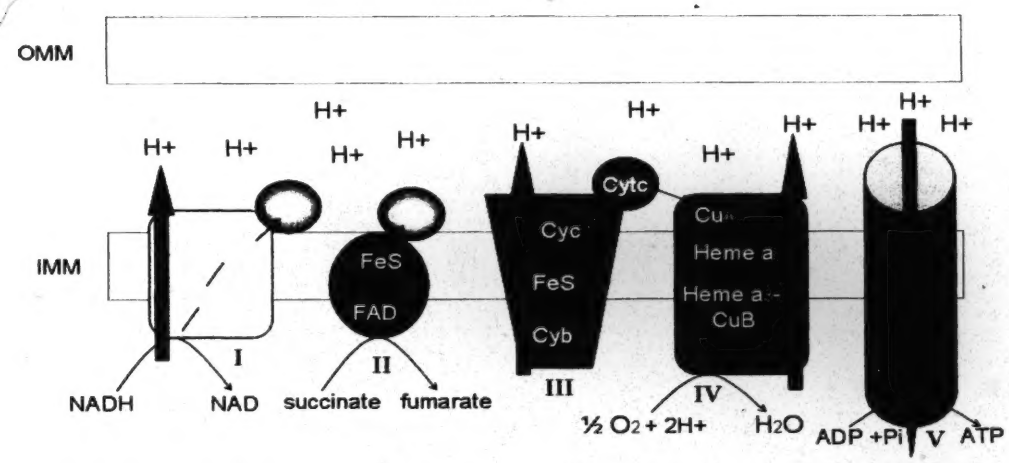
MITOCHONDRIA

Mitochondria make up 25 percent of a eukaryotic cell's cytoplasmic volume and play a part in many cellular functions, including energy production and cell death signaling pathways (Lodish, 2000). Mitochondria are composed of two membranes: the outer mitochondrial membrane and the inner mitochondrial membrane. The outer mitochondrial membrane (OMM) is composed of 50% protein and 50% lipids and is permeable to molecules with a molecular weight less than 10,000 kDa. The inner mitochondrial membrane (IMM) is composed of 20% lipid and 80% protein and forms cristae that extend into the matrix compartment. The IMM is also the site of the energy (adenosine triphosphate (ATP))-producing mitochondrial respiratory chain (MRC) (Lodish, 2000), which, in humans, has been proposed to transport 50-60 kg of ATP per day (Reichert and Neupert, 2004).Within the mitochondrial matrix, there exists a super-coiled, circular 16,569 basepair (bp) genome that contains 37 genes (Burger et al., 2003; Gibson, 2005). There are thought to be 2-10 copies of mtDNA per mitochondrion and as many as 10^3-10^4 identical copies of mtDNA in the mitochondria of a single cell. The mitochondrial genome encodes 13 protein subunits of the MRC complexes and 22 tRNAs and 2 rRNAs (Scheffler, 1999).

ELECTRON TRANSPORT CHAIN

Eukaryotic oxidative phosphorylation (OXPHOS) occurs in the mitochondrial inner membrane and involves five protein-lipid enzyme complexes (Figure 1-1) (Hatefi, 1985). The first four complexes form the electron transport chain and with the exception of complex II, collectively function to create an electrochemical gradient across the IMM, which is utilized by the fifth complex to produce energy in the form of ATP. Electrons are transferred through redox centers in each of the ETC complexes to the terminal electron acceptor located in complex IV, which reduces molecular oxygen to water. The complexes of the ETC include: NADH:ubiquinone oxidoreductase (complex I),

succinate:ubiquinone oxidoreductase (complex II), ubiquinol: ferricytochrome *c* oxidoreductase (complex III), ferrocytochrome *c*:oxygen oxidoreductase (complex IV) and ATP synthase (complex V). Collectively, there are approximately 83 structural subunits encoded by both the nuclear (nDNA) and mitochondrial (mtDNA) genomes (Marieke et al., 2001). Mutations in the structural subunits encoded by the mtDNA or nDNA can lead to conformational defects in the complexes. In addition, mutations in assembly factors encoded by the nDNA can lead to misassembly of the complexes and result in OXPHOS disorders in humans.



Complex	I	II	III	IV	V
nDNA subunits	43+	4	10	10(bovine)9(yeast)	10-16
mtDNA subunits	7	0	1	3	2
Redox centers	FMN, iron-sulfur	FAD, iron-sulfur	Cytochrome b, c, iron-sulfur	Copper, heme a	-

Figure 1-1 : *Mitochondrial Respiratory Chain*

The mitochondrial respiratory chain is composed of five multi-subunit complexes. Each of the first four complexes transfers electrons through a series of oxidation and reduction events to the terminal electron acceptor located in complex IV, which is responsible for the reduction of oxygen to water. Electron transport through complex I, III and IV is coupled to proton pumping across the inner membrane, which creates a proton gradient across the IMM. Complex V (ATP synthase) utilizes the proton gradient for the production of ATP from ADP and inorganic phosphate. ATP is transported to many different locations within the cell for various energy-requiring events. The table above indicates the number of mitochondrial-encoded and nuclear-encoded subunits, as well as the redox centers required for electron transport within each complex.

CYTOCHROME OXIDASE

Complex IV, also known as cytochrome oxidase (COX), is the terminal electron acceptor of the ETC. COX functions in dimeric form to catalyze the transfer of electrons from cytochrome *c* to molecular oxygen. In mammals, the COX monomer is composed of three mtDNA-encoded subunits and 10 nDNA-encoded subunits (9 nDNA-encoded subunits in yeast). The mitochondrial encoded subunits form the catalytic core of the enzyme and contain four redox-active components. The COX I subunit contains two heme A sites and the Cu_B site, while COX II contains the binuclear Cu_A site (Moraes et al., 2004). Zinc and magnesium ions are also present within the mature holoenzyme, but are not redox-active components of the enzyme. COX from the bacterium *Paracoccus denitrificans* (Iwata et al., 1995) and from bovine heart mitochondria (Tsukihara et al., 2003) have been resolved by X-ray crystallography. These crystal structures have provided key information on the locations of the redox centers within the subunits and have helped in understanding the conformational changes that occur during electron transfer and proton pumping.

Structure

Once the COX structure from bovine heart mitochondria was resolved, the placement and functional importance of COX subunits became clear (Figure 1-2) (Tsukihara et al., 1995). Twelve transmembrane helices of COX I span the IMM in a four, three-arm spiral pattern that serves as a scaffold for hemes A and Cu_B (Schultz and Chan, 2001). Subunit II contains two transmembrane helices, a large extramembranous domain capping the enzyme and a cuprodoxin fold motif (Schultz and Chan, 2001). Subunit III is composed of seven transmembrane helices and is essential for COX function, but its precise functional role is unclear. A total of 10 nuclear-encoded subunits surround the catalytic core of the enzyme. Each of the nuclear subunits, IV, VIa, VIc, VIIa, VIIb, VIIc and VIII, contain single transmembrane helixes that surround subunits I and III (Table 1-1) (Schultz and Chan, 2001). Subunits Va and Vb are extramembranous on the matrix side

and subunit VIb is extramembranous on the cytosolic side of the IMM. Altogether, the nuclear-encoded subunits are thought to control COX activity by regulating respiration rates and proton coupling (Grossman and Lomax, 1997).

Electron Transport and Proton Translocation

The redox-active sites within COX are required for electron transfer and proton pumping (Tsukihara et al., 2003). For electron transport to occur, each of the copper and heme sites must be assembled correctly within the catalytic core of COX. Electron transfer begins at the Cu_A site (subunit II), which receives a single electron from cytochrome *c*, after cytochrome *c* situates at the docking site of the holoenzyme on the IMS side of the membrane (Brunori et al., 2005; Faxen et al., 2005). The Cu_A site contains two copper atoms bridged between two cysteine residues of subunit II. A magnesium ion, embedded between subunit I and II, has been proposed to bridge the Cu_A site with the heme A_3 site through a hydrogen-bond network, which aids in electron transfer or acts as a gated ion channel for proton pumping (Tsukihara et al., 1995). Heme A is located between two histidines within two transmembrane helices of subunit I. Subunit I contains two redox-active sites, heme A and heme A_3-Cu_B and two proton-conducting pathways composed of hydrophilic amino acids. These amino acids create water-permissive hydrogen-bonded networks (Fattoretti et al., 2004). Following the reduction of heme A, electrons are transferred to the heme A_3-Cu_B site and subsequently to O_2 (Gennis and Ferguson-Miller, 1995; Brunori et al., 2005). The free energy produced during electron transport is adequate to break the bond between dioxygen, thus reducing oxygen to water. Electron transport is coupled to proton translocation, which contributes to the electrochemical gradient utilized by ATP-synthase for the production of ATP. There are three proton pathways that pump protons across the IMM and provide O_2 with protons for water formation (Tsukihara et al., 2003). The proton pump mechanism understood today is explained by conformational changes that occur in COX during oxidation and reduction at the redox-centers.

Table 1-1 : *Comparison of yeast and bovine COX subunits*

Nomenclature differences between yeast and bovine COX subunits.

Yeast	Bovine
1	I
2	II
3	III
5a	IV
6	Va
4	Vb
9	VIb
6a	VIa
7a	VIc
7	VIIa
12	VIIb
8	VIIc
	VIII

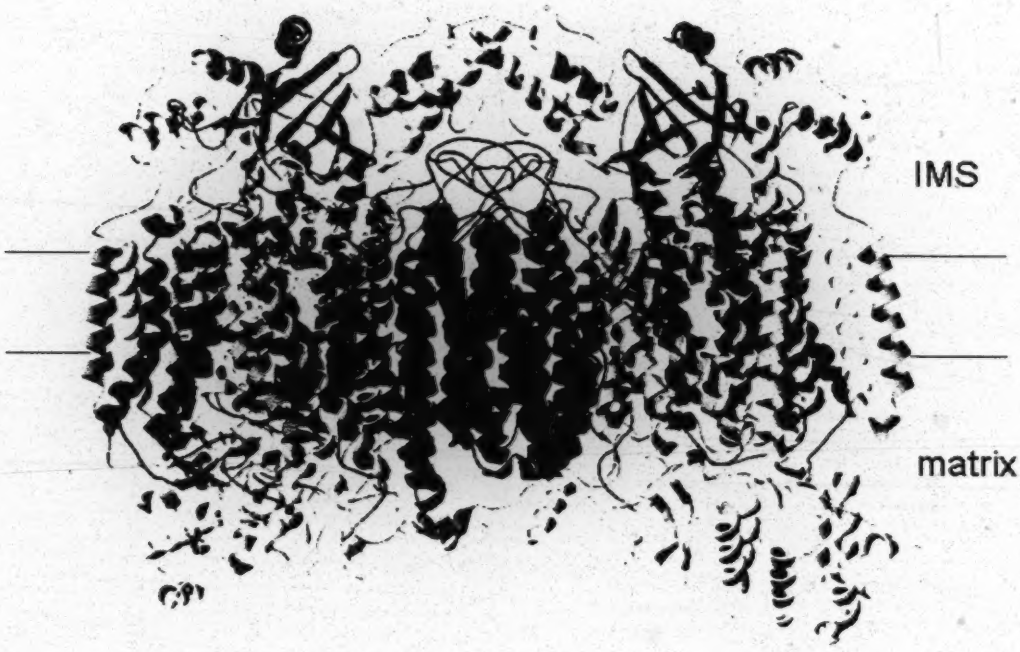


Figure 1-2 : C*rystal structure of bovine cytochrome c oxidase at the fully oxidized state*
COX functions as a dimer in the inner mitochondrial membrane, as shown above. The crystal structure was obtained from Entrez PubMed (PDB #: IV54) and was modified using Swiss PDB Viewer (version 3.7). Each colour represents a different subunit; COX I – orange, COX II – maroon, COX III – red, COX IV – yellow, COX Va – light green, COX Vb – light blue, COX VIa – pink, COX VIb – brown, COX VIc – turquoise, COX VIIa – grey, COX VIIb – dark green, COX VIIc – dark blue, COX VIII – purple. The metal and hemes are not visible in this diagram.

CYTOCHROME OXIDASE ASSEMBLY

Petite mutants

Mitochondrial cytochrome oxidase assembly has been thoroughly studied in the yeast, *Saccharomyces cerevisiae*. *S. cerevisiae* is a facultative anaerobe able to maintain its energy requirements either by fermentation or respiration (Tzagoloff and Dieckmann, 1990). The ability of yeast to respire or ferment has provided researchers with an ideal genetic and biochemical model for the study of mitochondria, and in particular, for the study of cytochrome oxidase assembly. In non-permissive environments, such as ethanol or glycerol, yeast are required to respire in order to grow. Respiratory deficient strains, however, are incapable of growth on non-fermentable carbon sources, but are capable of growth on the fermentable carbon source, glucose. Typically, respiratory deficient strains grow as small colonies on fermentable carbon sources when compared to wild-type yeast colonies. This morphological observation is termed a nuclear *petite* mutant and is used to describe respiratory deficient yeast strains with nuclear gene mutations. Over 2000 *pet* mutant strains have been investigated and grouped into 215 complementation groups, based on genetic and phenotypic analyses (Tzagoloff and Dieckmann, 1990). Genetic and biochemical screens of *pet* mutants have helped unfold the complex process of cytochrome oxidase assembly. Factors required for COX assembly act at both a transcriptional and translational level to organize the nuclear and mitochondrial-encoded subunits within the IMM. A subset of assembly factors are required for the placement of the redox-active components within COX during assembly.

Transcription and translation of cytochrome oxidase subunits

Controlled expression and translation of nuclear and mitochondrial-encoded subunits is important for regulating COX assembly. Under highly aerobic conditions, the requirement for COX is increased. Thus, increased expression and translation of COX subunits promotes an increase in COX assembly and activity. Transcription factors are important for transcriptional regulation of all nuclear-encoded COX subunits of rat

neurons. The transcription factor NRF-2 (or GA-binding protein – GABP) is a multi-subunit transcription factor that belongs to the E26 transformation-specific family of proteins, which bind GGAA core DNA sequences (Ongwijitwat and Wong-Riley, 2005). An NRF-2 promoter binding site has been identified upstream of all nuclear-encoded COX subunits, and acts as the only known transcriptional regulator of all ten nuclear-encoded COX genes. Interestingly, NRF-2 also regulates human, mouse and rat mitochondrial transcription factor A (mtTFA), which is responsible for the transcription of *COX I - III*. Taken together, NRF-2 may be critical for coordinating transcription of all 13 cytochrome oxidase subunits. To date, no investigation has identified a functionally homologous yeast Nrf-2p transcription factor that controls the transcription of yeast mtTFA and yeast nuclear-encoded subunits. Several yeast studies have attempted to identify transcription binding sites upstream of nuclear-encoded COX subunits. Many have found upstream GC-rich sites commonly found in ubiquitously expressed genes. The regulation of transcription binding sites, in particular for *COX5B* and *COX7C*, occurs by recognition of promoter sequences by the transcription factor, Sp1 (Grossman and Lomax, 1997). Sp1 is known to interact with basal promoter elements during transcription initiation. Another transcription factor, YY1, has also been found to regulate expression of these subunits. Other transcription factors for COX subunits have been identified in other species, which have been reviewed elsewhere (Lenka et al., 1998).

Translation of yeast COX subunits 1 – 3 occurs in the mitochondrial matrix and is dependent on mRNA-specific translational activators that recognize targets in the 5' untranslated region (Fiori et al., 2005) and mediate interactions between the mRNA transcript and mitochondrial ribosomes (Naithani et al., 2003). *COX1* is activated by Pet309p (Manthey and McEwen, 1995), *COX2* is activated by Pet111p (Green-Willms et al., 2001) and *COX3* is activated by Pet54p, Pet122p and Pet494p (Brown et al., 1994). Each of these activator proteins are integral inner membrane proteins thought to localize the translation of *COX1-3* to a similar location at the IMM. Translation of *COX1 – 3* on the matrix side of the IMM facilitates the assembly of the enzyme's catalytic core. Physical interactions between Oxa1p, an inner membrane translocase and mitochondrial ribosomes suggest that transcriptional activators direct the synthesis of COX1 – 3 to

Oxa1p for protein insertion into the IMM (Fiori et al., 2005). Two other proteins, Mss51p and Cox14p were recently found to control translation of *COX1*. Cox14p represses *COX1* translation by blocking access of the mRNA transcript to mitochondrial ribosomes or to translational activators, such as Mss51p (Barrientos et al., 2004).

The majority of COX subunits and all of the assembly factors are nuclear-encoded and must be transcribed, translated and imported into mitochondria. Nuclear-encoded subunits and assembly factors are synthesized as precursor proteins in the cytoplasm and targeted to the mitochondria either by an N-terminal targeting sequence or by an internal sequence. N-terminal targeting sequences of precursor proteins are proteolytically removed by a mitochondrial processing peptidase during or after import into the mitochondria. Other proteins contain internal targeting sequences that are not cleaved after mitochondrial import. As well, small IMS proteins have recently been found to import by a disulfide relay system that recognizes Cx_9C motifs. This mechanism of import will be discussed further below. Import across the OMM is mediated by the TOM (translocase of the OMM) complex and insertion of proteins into the IMM is facilitated by the TIM (translocase of the IMM) complex. Following import, proteins undergo a number of maturation events, including proteolytic removal of the mitochondrial targeting sequence, sorting to the appropriate membrane or compartment, folding, and assembly into their functional structures.

Formation of subcomplexes

Once the COX subunits are transcribed and translated, there are accessory proteins required to coordinate the assembly of the holoenzyme. These accessory proteins are essential for maturation of COX by aiding in subunit assembly and insertion of heme A and metal ions. The most complete model of COX assembly has been identified in mammals and is described by the formation of three subcomplexes (Figure 1-3) (Nijtmans et al., 1998). COX I forms the first subcomplex, S1, which obtains its redox-active sites, heme and copper, following insertion into the IMM (Shoubridge, 2001). The second subcomplex, S2, consists of COX I and COX IV and is supported by the bovine

COX crystal structure, which demonstrates a tight association between COX I and COX IV (Tsukihara et al., 1996). The third subcomplex, S3, consists of all of the subunits, except the peripheral subunits VIa and VIIa or VIIb likely incorporated at a late step in assembly (Nijtmans et al., 1998). The final complex is the fully assembled COX holoenzyme. Although only three subcomplexes have been identified, it is possible others exist, but have not yet been identified.

Pet100p is a *Saccharomyces cerevisiae* assembly facilitator located in the IMM (Forsha et al., 2001). It is found in a subcomplex, referred to as Complex A, of COX subunits 7, 7a and 8 and is thought to incorporate these subunits into the COX holoenzyme (Forsha et al., 2001). The C-terminal domain of Pet100p is required for association with Complex A. Two other subcomplexes have been identified in *pet100* null mutants and are referred to as Complex A' and Complex B. Complex A' is the same as Complex A, but lacks Pet100p and Complex B is composed of subunits Va and VI (Church et al., 2005). The formation of these subcomplexes is thought to stabilize the nuclear-imported subunits before their addition to the catalytic core. Subunits 1-3 are rapidly degraded in the absence of nuclear-encoded subunits, suggesting the importance for subcomplexes to be 'ready' for addition to the holoenzyme for structural stability. To date, no other yeast subcomplexes have been described.

Heme A formation and insertion into COX

Heme is a prosthetic group that consists of an iron atom embedded in a porphyrin ring, coordinated by four nitrogen atoms. Heme biosynthesis is a multistep process that begins with the formation of a porphyrin ring from succinyl-CoA and glycine. There are three distinct biologically important forms of heme (A, B and C), each of which is a part of the ETC complexes. Heme A, B, and C differ by modifications of the poryphryin ring. Heme B (protoheme), an important prosthetic group in myoglobin and hemoglobin, is the most common form of heme and is found in complex III of the ETC. Heme C is found in cytochrome *c* and cytochrome c_1 (in complex III) and heme A is found in complex IV of the ETC.

The production and insertion of heme into COX redox centers is essential for COX function. Heme A biosynthesis is carried out by Cox10p and Cox15p (Figure 1-4). *S. cerevisiae COX10* was identified by comparison to a bacterial cytochrome oxidase operon containing a highly homologous farnesyl transferase named *cyoE*. Cox10p is a 51 kDa farnesyl transferase that catalyzes the first step in heme A biosynthesis by farnesylation of the vinyl group at C2 of protoheme (heme B), producing the intermediate heme, heme O (Glerum and Tzagoloff, 1994). Although heme O can act as a prosthetic group in many bacterial terminal oxidases, it is non-functional as a prosthetic group in mitochondrial cytochrome oxidases and must be further modified to heme A. It is likely that the conversion of heme O to heme A is carried out in two steps involving the oxidation of the C8 pyrrole methyl moiety to an alcohol, and further oxidation to an aldehyde by a dehydrogenase. These reactions are carried out by Cox15p, ferredoxin (Yah1p) and ferredoxin reductase (Arh1p). In *Schizosaccharomyces pombe*, Yah1p has been found to be fused to Cox15p, suggesting that ferredoxin is important for heme A biosynthesis (Moraes et al., 2004). Ferredoxin is central in the biosynthesis of iron-sulfur clusters but is also thought to provide the reducing equivalents required for a monooxygenase reaction during the conversion of heme O to heme A. Once heme A has formed, it is ready for insertion into two hydrophobic pockets within Cox1p. The mechanism of heme A insertion into Cox1p, whether protein-dependent or not, has not yet been determined.

Copper delivery and insertion into COX

Copper is an essential metal ion required for many cellular processes, as well an important prosthetic group in COX. The delivery and insertion of copper to the Cu_A and Cu_B sites of COX is thought to be carried out by Cox17p, Sco1p and Cox11p. Copper placement in COX is essential for its function and activity. These COX assembly factors will be discussed in greater detail below.

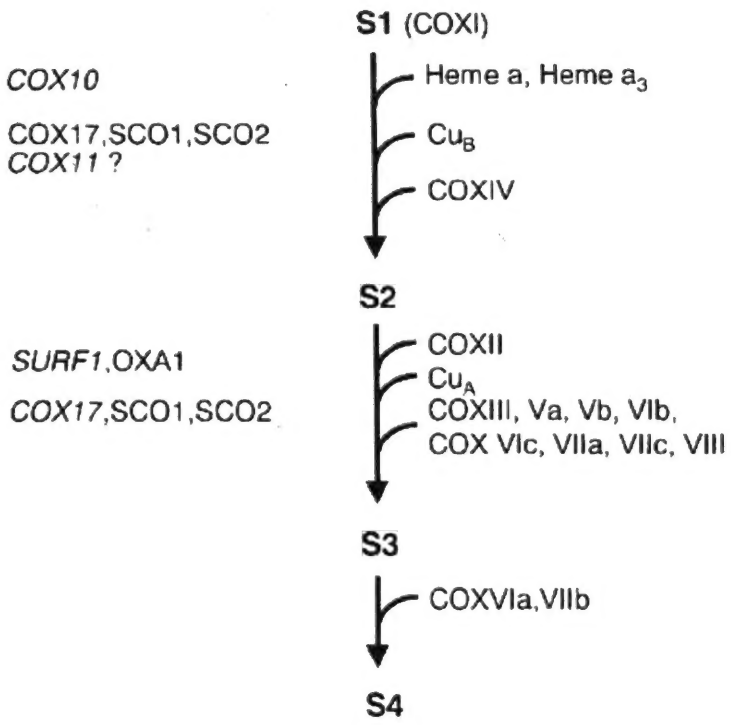


Figure 1-3 : *Mammalian COX assembly.*

The assembly of COX is thought to proceed through the formation of subcomplexes. The three subcomplexes identified form a preliminary model of COX assembly (Shoubridge, 2001).

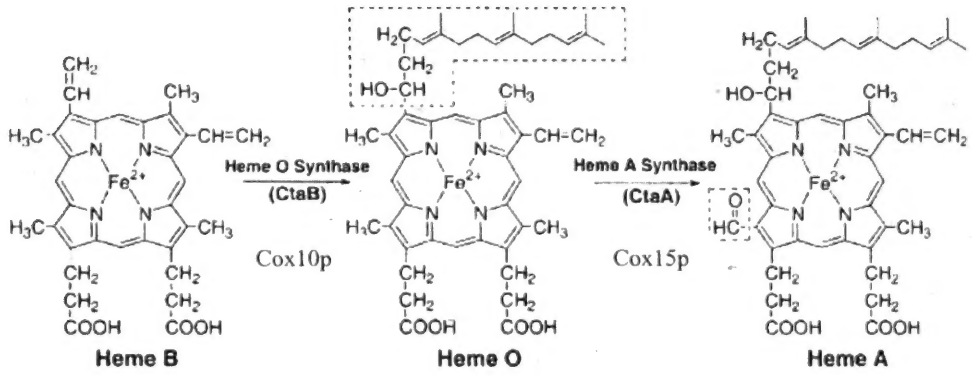


Figure 1-4 : *Biosynthesis of heme A.*

The synthesis of heme A in yeast is carried out by Cox10p and Cox15p. Cox10p is a farnesyl transferase that converts protoheme (heme B) to heme O and is homologous to the bacterial enzyme CtaB. The conversion of heme O to heme A is carried out by yeast Cox15p or its bacterial homolog CtaA. Once heme A is formed it is ready for insertion into the heme A sites of COX. Modified from (Brown et al., 2002).

Other assembly factors

The requirement of over 30 COX assembly factors involved in COX biogenesis demonstrates the complexity in forming a functional holoenzyme. Many COX assembly factors have been identified, but their exact functions are yet to be elucidated. Some of these assembly factors include Cox16p, Cox18p, Cox19p, Cox20p, Cox23p, Pet191p, Pet117p, and Sco2p. The assembly factors thought to be involved in metal transport include Cox19p (Nobrega et al., 2002), Cox23p (Barros et al., 2004) and Sco2p (Glerum et al., 1996b). Cox18p (Saracco and Fox, 2002; Souza et al., 2000) and Cox20p (Hell et al., 2000) are thought to stabilize Cox2p at a late stage in assembly. The roles of Cox16p (Carlson et al., 2003), Pet191p (McEwen et al., 1993) and Pet117p (McEwen et al., 1993) have yet to be determined.

ROLE OF COPPER IN CYTOCHROME OXIDASE ASSEMBLY

Cellular copper

Copper is an essential metal ion required for growth, development and survival in all organisms ranging from bacteria to humans (Linder, 1991). Copper functions as a prosthetic group for electron transport reactions in many different enzymatic pathways (Pena et al., 1999). Some proteins that require copper in order to function include: tyrosinase for melanin synthesis; Cu/Zn superoxide dismutase for antioxidant defense; dopamine β-hydroxylase for catecholamine biosynthesis; ceruloplasmin for iron homeostasis; and cytochrome oxidase for respiration (Waggoner et al., 1999). Copper exists in two different forms: Cu(II) and Cu(I). Cu(II) is more soluble and less toxic than Cu(I), whereas Cu(I) is highly reactive in the presence of oxygen or other electron acceptors and can initiate the production of reactive oxygen species (ROS) (Arredondo and Nunez, 2005). Tight coupling of copper to proteins can prevent the production of such harmful species within the cell and can prevent intracellular copper toxicity. Several mechanisms of copper transport and homeostasis have been identified, which include three distinct cellular copper transport pathways. These transport pathways were initially identified in the yeast, *Saccharomyces cerevisiae*. Yeast homology studies have also lead to the identification of the mammalian genes required for copper transport. The study of copper transport diseases has been advanced due to the similarity of copper homeostasis and transport in yeast and humans.

Yeast contain approximately 5×10^5 atoms of copper per cell depending on growth conditions, such as fermentation or respiration (Cobine et al., 2004). It is widely accepted that copper is not free within the cytosol of the cell, but rather is bound to proteins. Because of the toxicity and reactivity of cellular copper (Dameron and Harrison, 1998), it was accepted that this theory applied to mitochondrial copper pools as well. However, the mitochondrial matrix appears to contain soluble, anionic, low molecular weight complexes of copper (Cobine et al., 2004). It has been proposed that the pool of free copper in the mitochondrial matrix acts as a cellular buffering system. This was proposed because matrix copper levels were highly dependent on cytosolic copper levels.

However, it is unclear what consequences this may have for the cell, as mitochondria produce a high proportion of reactive oxygen species capable of reacting with copper. Such reactions result in lipid peroxidation, protein oxidation and DNA and RNA damage. Other protein-specific mechanisms of copper buffering exist within the cell and will be further discussed below.

Cellular uptake of copper

Copper uptake from the extracellular space is carried out by the Ctr1 family of proteins. Members of the Ctr1 family in yeast, plants and mammals contain three putative transmembrane domains that span the plasma membrane and an N-terminal rich in the methionine motifs, MxxM and MxM (Puig et al., 2002). These motifs are proposed to play a role in copper binding or copper capture on the extracellular side of the plasma membrane. Ctr1 members have a C-terminal rich in charged amino acids and several cysteine and histidine residues that allow for copper binding on the cytosolic side of the plasma membrane. Yeast contain two high-affinity copper transporters, Ctr1p and Ctr3p, and one low-affinity copper transporter, Ctr2p. Prior to copper uptake at the plasma membrane, Cu(II) is reduced to Cu(I) by the cell surface Fe(III)/Cu(II) reductases, Fre1p and Fre7p.

A long-known link between copper availability and iron transport was explained once the high-affinity yeast copper transporters, Ctr1p and Ctr3p, were identified. The *CTR1* gene was identified in mutants defective in iron transport (Labbe et al., 1999) due to a lack of copper insertion into the high-affinity iron transporter, Fet3p (Pena et al., 1999). The *CTR3* gene was identified as a suppressor of a copper starvation phenotype observed in a mutant *ctr1* strain (Pena et al., 1999) demonstrating functional similarities between Ctr1p and Ctr3p. A disruption in the *ctr1* gene results in poor growth in copper-depleted media, respiratory deficiency, oxygen sensitivity, inability to activate metallothionein and the absence of high affinity iron transport. All of these phenotypes are explained by the copper transport pathways present in the cell (Figure 1-5). These

include copper transport to COX, to the high-affinity iron uptake protein Fet3p, and to Cu,Zn superoxide dismutase (SOD1), which all play unique cellular roles.

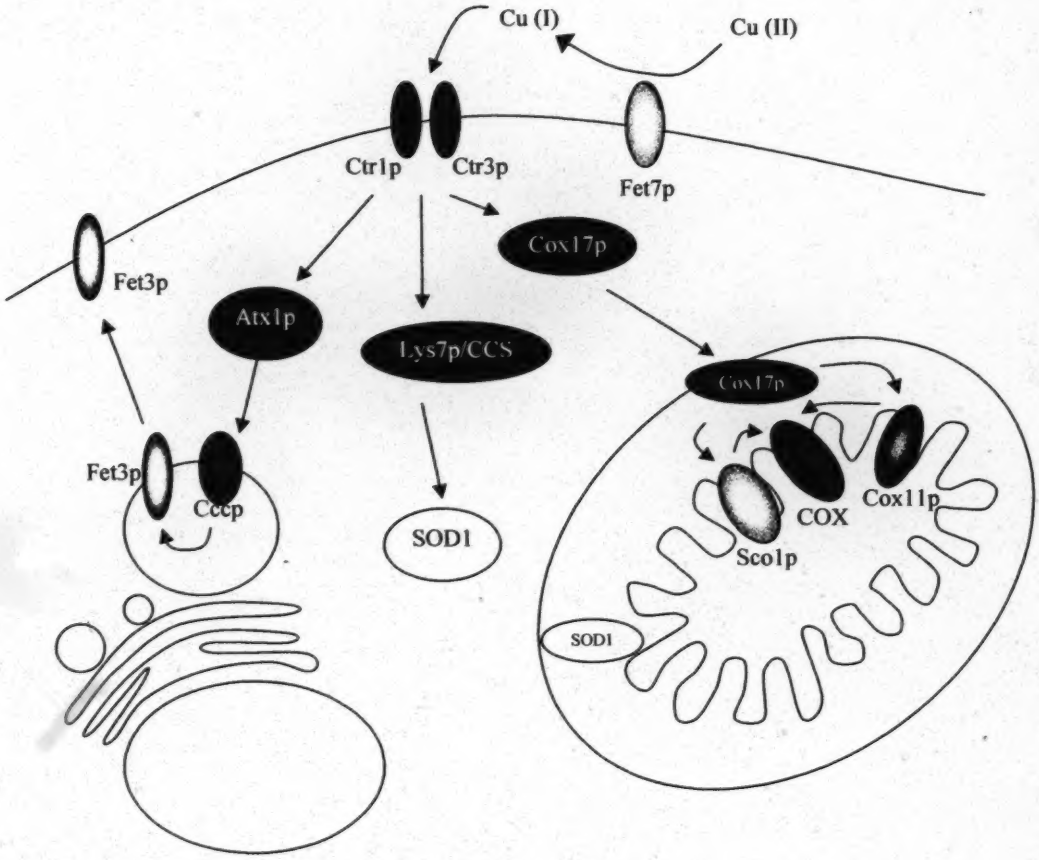


Figure 1-5 : *Cellular copper transport pathways.*

Copper is reduced at the plasma membrane by Fet7p and transported across the plasma membrane as Cu(I) by the high affinity copper transporters, Ctr1p and Ctr3p. Three copper metallochaperones transport copper via three distinct pathways in the cell, directing copper to the mitochondria, secretory pathway and to Cu, Zn superoxide dismutase.

Copper transport to the secretory pathway and Cu, Zn Superoxide Dismutase

There are three copper transport pathways that follow uptake of copper by Ctr1p or Ctr3p (Figure 1-5). The transport of copper to the high affinity iron transporter, Fet3p, requires the copper chaperone, Atx1p and the copper transporter, Ccc2p. Atx1p was originally identified as a putative antioxidant molecule that suppressed oxidative damage in cells lacking Sod1p (Lin and Culotta, 1995; O'Halloran and Culotta, 2000; Field et al., 2002). Atx1p is a cytosolic peptide of approximately 8.2 kDa that binds one Cu(I) atom as a 2- or 3-coordinate complex, with thiol ligands from two conserved cysteines located in a surface accessible loop in the first α-helix of the $\beta\alpha\beta\beta\alpha\beta$ 'ferrodoxin-like' fold (Pena et al., 1999; Askwith and Kaplan, 1998; Huffman and O'Halloran, 2001). Cu-Atx1p docks with the N-terminal of Ccc2p at the trans-Golgi network (Huffman and O'Halloran, 2001). Ccc2p is a P-type adenosine triphosphatase (ATPase) that contains an MxCxxC metal-binding domain on its N-terminus (Valentine and Gralla, 1997). By yeast two-hybrid screens, Atx1p was found to associate with Ccc2p through structurally similar domains. This interaction is dependent on a 2-3 copper bridged complex that allows for the incorporation of copper into Ccc2p (Labbe et al., 1999). After dissociation from the complex, Atx1p is copper-free (Huffman and O'Halloran, 2001). By ATP hydrolysis, Ccc2p pumps copper into a late Golgi secretory compartment and with the aid of Gef1p, incorporates copper into Fet3p (Labbe et al., 1999). Fet3p is a high affinity iron transporter that is secreted to the plasma membrane (Askwith and Kaplan, 1998). It contains a multicopper oxidase domain that is concomitantly able to oxidize substrates with the reduction of oxygen to water and convert ferrous iron to ferric iron by its ferroxidase activity.

Human homologs for each of these proteins have been identified and are known to be associated with human disease (Waggoner et al., 1999; Harris, 2000). The human protein, Hah1, complements yeast Atx1p function by transporting copper to P-type ATPases through N-terminal interactions with their metal-binding domains (Andrews, 2001). A defect of the P-type ATPase, ATP7A, is associated with Menkes disease (Horn et al., 1984; Mercer et al., 1993; Vulpe et al., 1993; Medeiros and Jennings, 2002). In

Menkes disease, copper transport is primarily defective in the intestine, thus copper uptake and transport to other tissues is prevented, resulting in an overall copper deficiency (Askwith and Kaplan, 1998). Menkes disease is usually lethal, presenting with premature delivery, low birth weight, hypothermia, hypotonia, diarrhea, developmental delay and seizures (Cox, 1999). Various types of mutations and deletions of *ATP7A* are known to cause a Menkes phenotype. A second P-type ATPase is associated with human Wilson disease, which is a result of mutations in the gene product of *ATP7B* (Tanzi et al., 1993; Cox et al., 1972). Mutations in ATP7B can prevent cellular copper export and result in copper accumulation. This can have a profound effect on many tissues, but especially on the brain and liver tissues (Askwith and Kaplan, 1998). Clinical manifestations include: neurological, psychiatric and hepatic. Molecular defects of Wilson disease result in a reduction of copper excretion into the bile and the failure to incorporate copper into ceruloplasm (Cox, 1999). Ceruloplasm is the human form of yeast Fet3p, but functions slightly different. In yeast, Fet3p transports iron into the cytoplasm, however, in humans, ceruloplasmin releases and transports iron through the body.

A second copper transport pathway results in incorporation of copper into Cu, Zn superoxide dismutase (Sod1p) by the yeast metallochaperone Lys7p. Sod1p functions to convert superoxide radicals into hydrogen peroxide. Cellular degradation of superoxide radicals is important in order to prevent lipid peroxidation and DNA damage (Labbe and Thiele, 1999). Mitochondria produce the majority of cellular superoxide radicals during oxidative respiration. Almost 2% of consumed oxygen is incompletely reduced in mitochondria resulting in the production of reactive oxygen species (ROS), such as the superoxide anion (Sturtz et al., 2001). Sod1p utilizes the redox properties of copper in order to catalyze degradation of superoxide (O_2^-) to hydrogen peroxide and water (Puig et al., 2002). Sod1p is a soluble homodimer located in the cytosol and mitochondrial intermembrane space. Although mitochondria contain only 1-2% of total cellular Sod1p, the amount of Sod1p per volume is just as high in the IMS as in the cytosol (Field et al., 2002). Mutations in human *SOD1* cause amylotrophic lateral sclerosis (ALS), also known as Lou Gehrig's Disease (De Freitas et al., 2003). The encoded mutant *SOD1* protein has

an altered conformation that leads to an increase in ROS production through its bound copper atom. This causes the neurodegenerative phenotype that leads to progressive muscular weakness, atrophy and death (Mercer, 2001).

The incorporation of copper into Sod1p requires the copper chaperone, Lys7p, in yeast and CCS, in mammals. *lys7* null yeast strains maintain wild-type levels of Sod1p, but lack Sod1p activity. Human CCS and yeast Lys7p contain three distinct functional domains. Domain I shares 34% similarity to Atx1p and contains an MxCxxC metal binding motif (Lamb et al., 1999; Puig et al., 2002) that is responsible for the acquisition of one copper ion (Huffman and O'Halloran, 2001). Domain II is shaped like a 'greek key' with a β-barrel containing a region structurally similar to a region of SOD1. The interaction of this domain with SOD1 facilitates copper delivery, but not copper acquisition. The third domain, located at the C-terminus, is unique to CCS-like proteins and is required for CCS activity and copper incorporation into SOD1. By yeast two-hybrid analysis, heterodimeric complexes of CCS and SOD1 have been identified and found to be dependent on a series of disulphide bridges that assist in copper transfer between the two proteins (Huffman and O'Halloran, 2001).

Copper transport to mitochondria for insertion into COX

Cox17p is a copper chaperone necessary for COX holoenzyme assembly and activity (Figure 1-6). *COX17* was first cloned in yeast by transformation of the mutant C129/U1, in complementation group G74 of *pet* mutants (Glerum et al., 1996a). The C129 mutant contains a C57Y amino acid substitution and is respiratory deficient, with a specific defect in complex IV of the respiratory chain. Cox17p is highly acidic, hydrophilic and contains seven conserved cysteine residues (Glerum et al., 1996a; Heaton et al., 2000). Growth could be restored in *cox17* null strains after supplementation with 0.4% copper in the growth media, suggesting Cox17p's role as a copper-binding protein (Glerum et al., 1996a). 60% of total cellular Cox17p resides in the mitochondria and 40% resides in the cytosolic space (Beers et al., 1997). The current model of copper delivery to COX involves Cox17p delivering copper to Cox11p and Sco1p for subsequent placement at the

copper redox-sites in the COX apoenzyme (Figure 1-6) (Glerum et al., 1996b). Cox17p is thought to transport 40% of its copper to Sco1p and 55% of its copper to Cox11p (Abajian et al., 2004).

In comparison to wild-type yeast strains, *cox17* point mutants have reduced steady-state Cox17p levels in both cytosolic and mitochondrial fractions and a selective loss of Cox2p, whereas *cox17* null mutants completely lose Cox1 - 3p. *cox17* point mutants have been placed into three phenotypically-distinct groups (Punter and Glerum, 2003). The first phenotypic group of mutants has a reduction in respiration, maintained levels of Cox2p and the presence of an absorption spectra of the mitochondrial aa_3 cytochrome, indicating mild COX instability. The second class of mutants has no COX activity or growth on non-fermentable carbon sources and lacks an aa_3 spectral peak. The third phenotypic group of mutants has a selective loss of Cox2p. All mutants however retain Cox1p, suggesting specific copper delivery to the Cu_A site in Cox2p, late in the COX assembly pathway.

Site-directed and random mutagenesis of *COX17* identified the amino acid residues required for copper binding and mitochondrial import. All seven cysteine residues in Cox17p have been mutated, which defined the copper-binding motif, CCxC, located at C23, C24 and C26 (Figure 1-7) (Heaton et al., 2000). When C23, C24 and C26 are mutated to serines, the mutants are unable to utilize non-fermentable carbon sources and have reduced COX activity. However, mutations of C16 and C36 to serines results in respiratory competent mutants with normal levels of COX activity. C23S and C24S double mutants do not bind copper, which further supported the role of these residues in copper binding. Cox17p binds Cu(I) in a solvent-shielded environment, in the copper-binding domain, CCxC (Heaton et al., 2001; Srinivasan et al., 1998; Abajian et al., 2004). The Cox17p NMR structure identified one copper atom bound by the copper-binding residues, C23 and C26, at a bond angle of 164 degrees (Abajian et al., 2004). Mutations at C47 and C57 allow Cox17p to bind copper, but prevent the accumulation of Cox17p in the IMS. Interestingly, the mutant, C47S, is respiratory competent (Heaton et al., 2000), but when this residue is mutated to arginine, it is respiratory deficient (Punter and Glerum, 2003).

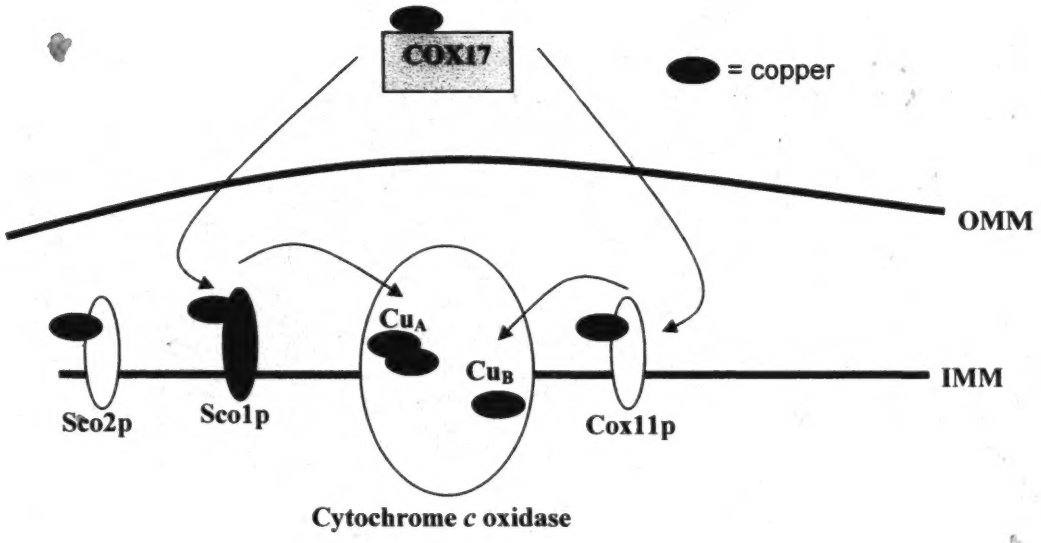


Figure 1-6 : *Copper transport to mitochondria.*

Cox17p is thought to deliver copper to Sco1p and Cox11p, which subsequently deliver copper to COX during assembly. The Cu_A site in COX is located within Cox2p and thought to be copper loaded by Sco1p and the Cu_B site is located within Cox1p and thought to be copper loaded by Cox11p. OMM = outer mitochondrial membrane. IMM = inner mitochondrial membrane.

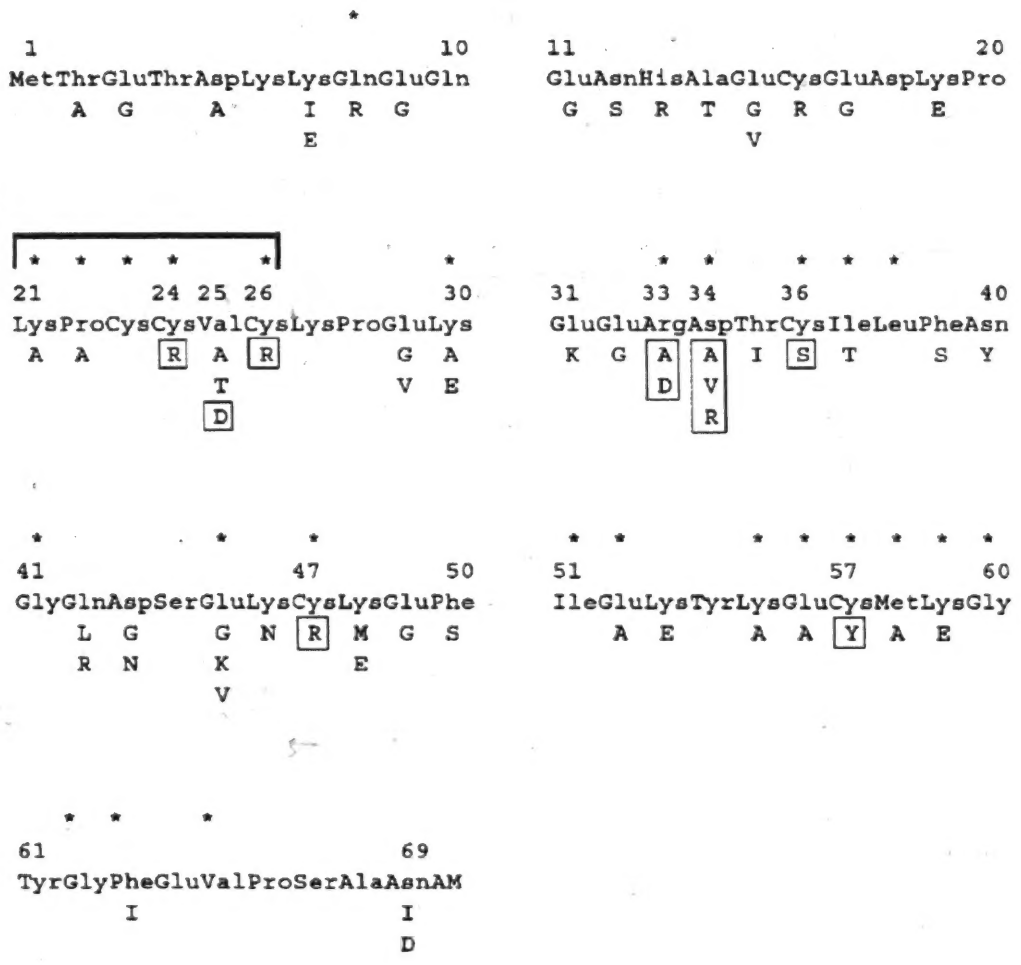


Figure 1-7 : M*utagenesis of yeast Cox17p*

The amino acid sequence of Cox17p is shown. The amino acids that have been mutated are shown below each residue. Those residues that lead to a respiratory deficient phenotype are boxed. The asterisk represents the residues that are conserved between yeast and humans. Figure obtained from Punter and Glerum, 2003.

Nearly 70% of Cox17p amino acids have been mutated; however only $1/6^{th}$ of all amino acids and only 40% of conserved amino acids examined lead to a respiratory deficient phenotype. Since several of the Cox17p residues can be mutated with few leading to respiratory deficiency, Cox17p was proposed to be a natively unfolded protein (Punter and Glerum, 2003), which was borne out by the Cox17p NMR structure. The NMR structure of yeast *apo*-Cox17 and Cu-Cox17p has provided key information on the secondary conformation of Cox17p and its role in copper delivery to Sco1p and Cox11p (Abajian et al., 2004). Cox17p has 20 unstructured residues at the N-terminus, followed by two alpha helices at residues 27-39 and 48-60 in *apo*-Cox17p and at residues 28-39 and 48-59 in Cu-Cox17p. Typical features of natively unfolded proteins include the ability to be highly mutated with little phenotypic effect, a large net charge and flexibility. Cox17p contains 18.8% glutamic acid residues and 15.5% lysine residues in comparison to the average protein that has 6.2% and 5.7% of these residues, respectively. Both glutamic acid and lysine are polar residues, however glutamic acid is acidic and lysine is basic. According to the NMR structure for Cox17p, nearly 40% of all residues are unstructured and thus fit the characteristics of a natively unfolded protein.

A controversy has existed regarding the number of copper ions bound to Cox17p. Several researchers have sought to determine whether Cox17p binds one, two, three or four copper ions per protein (Kako et al., 2004; Srinivasan et al., 1998; Heaton et al., 2001; Palumaa et al., 2004). The redox state of porcine Cox17 appears to be the determining factor for the number of bound copper ions. In its fully reduced state, porcine Cox17 binds four copper ions and in its partially oxidized state, Cox17 contains two disulfide bonds and binds one copper ion(s) (Palumaa et al., 2004). In its fully oxidized state, porcine Cox17 contains three disulfide bridges and does not contain any bound metals. Due to functional similarities across several species, it is likely that other species of Cox17p maintain a redox-dependent copper-binding ability.

A newly discovered mechanism for importing small IMS proteins into the mitochondria has been found to import Cox17p and Cox19p into the intermembrane space, with the assistance of Mia40p (Chacinska et al., 2004). Cox17p and Cox19p, along with other small IMS proteins, contain a twin CX_9C motif necessary for disulfide bond

formation with Mia40p during import (Mesecke et al., 2005). Once imported into the IMS, Mia40p assists in assembly of proteins into their mature conformation. The import of Cox17p into the IMS in an unfolded fashion suggests that Cox17p may bind copper after import into the IMS, presumably from the non-proteinaceous pool of copper in the mitochondrial matrix (Cobine et al., 2004). However, there is currently no evidence to explain how the non-proteinaceous pool of copper located in the matrix could metallate Cox17p in the IMS, as they are spatially distinct. If this model is correct, other copper chaperones must be responsible for supplying the mitochondrial matrix and IMS with copper.

Mammalian COX17

A human homolog of yeast *COX17* was identified through a human cDNA expression library screen in a yeast *cox17* null mutant (Amaravadi et al., 1997). The ability of human COX17 to complement a yeast *cox17* null strain indicated that human COX17 functions similarly to yeast Cox17p. Human COX17 is cysteine rich and contains the copper-binding domain, KxCCxC. The human *COX17* gene spans 8 kb on chromosome 3q13.1-q21 and is composed of three exons and two introns, of 2 and 5.5 kb, respectively (Punter et al., 2000). Human *COX17* shares 93% and 92% identity with pig and mouse *COX17* sequences, respectively (Punter et al., 2000). At the time of human *COX17* characterization, a *COX17* pseudogene (*COX17P*) was identified on chromosome 13q14-q21. *COX17P* was proposed to be a processed pseudogene since it lacked exon/intron organization. *COX17P* was assumed to be non-functional since *COX17P* was not expressed in lymphoblastoid cells and contained three amino acid changes in the proposed coding region. The proposed *COX17P* product contains nucleotide changes at 32 C→T, 53 A→G, 73 G→A and 96 G→A (silent), which leads to the amino acid changes P11L, K18R and A25T. The A25T amino acid change is located within the copper-binding domain and was thought to abrogate *COX17P* function if the gene were to be transcribed and translated.

Mouse Cox17, identified through EST databases, has a highly similar amino acid sequence to human COX17 (Nishihara et al., 1998). Immunfluorescence studies of mouse

Cox17 demonstrated localization at the perinuclear region and not in the mitochondria, which suggested a role for mammalian Cox17 in synthesis or secretion of neuropeptide hormones (Kako et al., 2000). Heterozygous *Cox17* mice (+/-) were healthy, of normal size and fertile despite having a 50% reduction in *Cox17* transcripts and a 20% decrease in COX activity (Takahashi et al., 2002). However, homozygous knockout mice (-/-) were embryonic lethal at day E8.5 to day E10 and had severely reduced COX activity prior to death. The mouse *Cox17* gene has the same genomic layout as human *COX17*, spanning 5.7 kb on chromosome 16 and containing three exons (Takahashi et al., 2001). Transcription is initiated at CpG islands, 80 bp upstream of the ATG site, by RNA polymerase II. As opposed to human COX17, which contains a single polyadenylation signal, mouse Cox17 contains two polyadenylation signals located at the 3' untranslated region (UTR).

SCO1 and SCO2

Yeast Sco1p was first identified in 1988 as an essential COX assembly factor important for Cox2p accumulation (Schulze and Rodel, 1988; Krummeck and Rodel, 1990). As well, a homolog, known as Sco2p, exists within the yeast genome. Sco1p and Sco2p are integral proteins of the IMM (Buchwald et al., 1991; Lode et al., 2002) that contain the metal binding domain CxxxC in the C-terminal IMS-protruding region (Glerum et al., 1996b). As a dimer, Sco1p is known to bind two copper ions. The postulation that Cox17p delivers copper to Sco1p and/or Sco2p for insertion into COX came from the finding that Sco1p was able to suppress a *cox17* null strain, implying a downstream role in copper transport (Glerum et al., 1996b). Sco2p was able to partially restore respiration in a *cox17* null mutant when provided with supplementary copper. Although Sco1p and Sco2p are highly homologous, they are not able to functionally complement one another, suggesting that they do not play identical roles in the cell. Knockout *sco1* strains are not able to utilize non-fermentable carbon sources and are COX deficient, whereas *sco2* knockout strains are respiratory competent with normal levels of COX activity. It has been suggested that Sco1p and Sco2p form a heterodimer, however, when a Sco1p/Sco2p chimeric protein is generated, it is non-functional (Rentzsch et al., 1999).

Immunoprecipitation analysis has identified interactions of Sco1p and Sco2p with Cox2p in a copper-independent fashion (Lode et al., 2000; Lode et al., 2002). In addition to evidence for Sco1p's role in copper delivery to COX, Sco1p and Sco2p have similarities to peroxiredoxins and thiol:sulfide oxidoreductases with a thioredoxin fold. This suggests that Sco1p and Sco2p may play a catalytic role in addition to a copper transport role (Chinenov, 2000). The similarity between Sco1p and peroxiredoxins was further supported upon the resolution of the human SCO1 crystal structure (Williams et al., 2005). Sco1p has since been proposed to act as a copper-dependent redox switch that oxidizes Cu(I) to Cu(II), permitting cysteines in the CxxxC copper-binding domain to participate in a peroxidase reaction. It is possible that Sco1p plays a dual role, both as a copper transporter and in redox signaling. Further research in defining the exact function of Sco1p will advance our understanding of Sco1p's role within mitochondria.

COX11

Yeast *cox11* mutants lack an aa_3 spectral peak, COX activity and have significantly decreased levels of steady-state Cox1p, Cox2p and Cox3p. Bacterial *cox11* null strains (*Rhodobacter sphaeroides*) have a blue shifted spectral peak and a specific loss of Cu_B. As well, *cox11* mutants have a 70-85% decrease in Cox1p and a reduction in magnesium between Cox1p and Cox2p (Hiser et al., 2000). Cox11p is thus predicted to be involved in copper delivery to Cox1p during COX assembly. Cox11p is an integral IMM protein (Carr et al., 2002) that contains an N-terminal targeting sequence, as well as a CFCF metal binding motif (Hiser et al., 2000). Cox11p binds one copper atom (Cu(I)) per monomer and is thought to receive copper from Cox17p, as determined *in vitro* by a copper transfer assay (Horng et al., 2004). The combination of *apo*-Cox11 and Cu-Cox17p leads to copper loading of Cox11p. Although no stable interactions between Cox11p and Cox17p have been identified, it is possible that transient interactions exist between the two. According to the Cox11p NMR structure from bacterial *Sinorhizobium meliloti*, the soluble IMS domain forms a copper-independent homodimer and a β-immunoglobulin-like fold (Banci et al., 2005). Further studies of Cox11p are necessary to clearly understand its functional role in mitochondria.

MITOCHONDRIAL DISEASE

Mitochondrial disease is a type of inborn error of metabolism that occurs in 1 in 10,000 live births (Smeitink et al., 2001). The disease phenotype usually presents itself within the first year of life and comes with a serious and most often fatal prognosis. Each of the five respiratory chain complexes have associated human deficiencies with a variety of identified mutations and an even greater number of unidentified mutations. In 2001, 100 point mutations and innumerable rearrangements were associated with mitochondrial disease (Smeitink et al 2001) and by 2004, there were 90 additional mutations identified (Pecina et al., 2004b). The mitochondrial genome encodes 13 respiratory chain subunits, all of which have been found to contain disease-causing mutations. The nuclear DNA is vast in its contributions to the MRC, and thus contributes to the majority of mutations found in mitochondrial disorders. Since the MRC is composed of more than 85 structural subunits organized by approximately 30 assembly factors, cooperation from both the nuclear and mitochondrial genomes is required for proper functioning (Smeitink et al., 2001). The high number of proteins involved in complex biogenesis has made the identification of disorders and their genetic causes difficult. As well, the vast clinical and genetic heterogeneity that presents with these disorders has limited diagnosis and treatment. Unfortunately, there are no cures for mitochondrial disease, however limited palliative, pharmacological and surgical interventions are available to alleviate symptoms. As a result of mitochondrial disease, many patients accumulate toxic substances, such as lactic acid, have a lack of electron acceptors, metabolites and cofactors and have increased damage by oxygen radicals. Treatment with vitamin K3, C, L-carnitine and Co-enzyme Q, which act as electron acceptors, metabolites and oxygen radical scavengers, respectively, has increased patients' quality of life.

One of the difficulties in understanding mitochondrial disease is the differences in mitochondrial genetics in comparison to typical Mendelian genetics. The first main difference is that mitochondria are maternally inherited. At fertilization, zygotic mitochondria are derived from the oocyte and lost from the sperm (DiMauro, 2001). Thus, mothers carrying a mitochondrial DNA mutation can transmit it to all their

progeny. However, only the daughters of that progeny can transmit it to the next generation. The second difference is polyplasmy, which refers to the presence of several copies (10^3 to 10^4) of mtDNA in each cell (Smeitink et al., 2001). The third difference, known as heteroplasmy, is the existence of two mtDNA populations: a wild-type and mutant population. The fourth difference, threshold effect, is exhibited by the fact that a critical number of mutant mtDNA are required to cause a respiratory chain defect. The threshold has been known to range from 20-85+%, depending on the tissue involved. Tissues that are highly dependent on the respiratory chain have a much lower threshold for mutant mtDNA, than tissues less dependent on the respiratory chain. Mitochondrial inheritance, polyplasmy, heteroplasmy and threshold effect contribute to the clinical heterogeneity observed in patients with mitochondrial disease.

Although a large number of mitochondrial disorders arise from defects in all of the respiratory chain complexes, I will be focusing on mitochondrial disease associated with cytochrome oxidase deficiency. Mutations in the three mitochondrial-encoded subunits and mutations in assembly factors critical for COX biogenesis and stability have been found to cause COX deficiency. However, many patients with COX deficiencies are yet to be characterized. The following two sections will cover COX deficiencies due to mtDNA mutations and COX deficiencies due to nDNA mutations. See Table 1-2 for a summary.

COX deficiency caused by mtDNA mutations

As discussed earlier, the three mitochondrial-encoded subunits form the catalytic core of cytochrome oxidase. Several different types of mutations from single pedigrees have been identified in all three mitochondrial-encoded COX subunits. The mutations identified to date have been heteroplasmic and their associated phenotypes have been tissue-specific and dependent on the load of mutated mtDNA.

COX I microdeletions and nonsense mutations have been identified in a few cases of COX deficiency. In one case, a 21-year old girl had a nonsense mutation in her blood, muscle and myoblasts, leading to a deletion of the latter 33% of the protein

Table 1-2 : *Genetic defects associated with human COX deficiency*

Gene	Altered function	Number of known mutations	Type of mutations	Clinical Phenotypes	References
Mutations in mtDNA associated with COX deficiency					
COX I	disruption in COX assembly	3	microdeletions, nonsense	COX deficiency, epilepsia partialis continua, motor neuron disease	(Bruno et al., 1999;Varlamov et al., 2002)
COX II	loss in COX II, failure to assemble COX holoenzyme	4	missense, microdeletion, nonsense	COX deficiency, proximal myopathy, lactic acidosis	(Clark et al., 1999; Rahman et al., 1999;Wong et al., 2001;Campos et al., 2001)
COX III	COX instability, impaired COX assembly, reduction in steady-state COX II, COX III	4	microdeletion, point mutation, insertion, frameshift	COX deficiency, exercise intolerance, lactic acidosis, encephalopathy	(Keightley et al., 1996;Hanna et al., 1998;Tiranti et al., 2000;Horvath et al., 2002)
Mutations in nDNA associated with COX deficiency					
SURF1	arrest COX assembly at S2 or S3	40	Insertion, deletion, missense, nonsense	Leigh Syndrome associated with COX deficiency, leukodystrophy	(Pecina et al., 2004a;Shoubridge, 2001; Barrientos, 2003)

LRPPRC	instability of mitochondrial COX mRNA transcripts	2	8-bp deletion, missense	Leigh Syndrome, French Canadian type, COX deficiency	(Mootha et al., 2003; Hou et al., 1994;Xu et al., 2004)
SCO1	prevent copper transport to COX subunit II, COX destabilization	2	Missense, 2bp-deletion	encephalopathy, ketoacidotic coma, hepatopathy	(Papadopoulou et al., 1999; Petruzzella et al., 1998; Valnot et al., 2000a)
SCO2	prevent copper transport to COX subunit II, COX destabilization	7	Nonsense, missense, 10bp-duplication	fatal infantile cardioencephalomyopathy, Leigh-like syndrome	(Papadopoulou et al., 1999; Tarnopolsky et al., 2004; Jaksch et al., 2001)
COX10	prevent conversion of protoheme B to heme O	3	Missense, nonsense	leukodystrophy, tubulopathy, fatal infantile hypertrophic cardiomyopathy and Leigh-like syndrome	(Valnot et al., 2000b;Antonicka et al., 2003a)
COX15	prevent conversion of heme O to heme A	2	Splice site, point mutation	fatal infantile cardioencephalomyopathy	(Antonicka et al., 2003b)

(Bruno et al., 1999). Fusion analysis with human cells lacking mtDNA demonstrated a relatively low threshold for the A→G transition at nucleotide 6930. Because of the low mutational threshold of this mutation, a disruption in assembly of the COX holoenzyme is likely. Clinical examination of this patient identified bilateral symmetric hyperintensities in the basal ganglia and cerebellar atrophy, severe visual defect with optic atrophy and deafness. A second case of a *COX I* mutation involved a missense mutation at C6489A in a 17-year-old patient with COX deficiency and epilepsia partialis continua (Varlamov et al., 2002). This heteroplasmic mutation replaced a highly conserved leucine residue with an isoleucine residue. Biochemical analysis demonstrated a decrease in COX activity and a 95% mutant threshold level to produce a COX deficiency. Other *COX I* mutations include a microdeletion associated with motor neuron disease and a nonsense mutation in a patient with myopathy. In general, mutations in *COX I* cause a reduction of COX II, COX III and several nuclear-encoded subunits, as well as a reduction in holoenzyme activity.

There have been few *COX II* mutations identified in patients with COX deficiency. In 1999, a family with a history of COX deficiency was found to contain a transition mutation in the initiation codon of *COX II* (T7587C), causing a methionine to threonine amino acid change (Clark et al., 1999). Affected family members included a 57-year-old female index case and her 34-year-old son. The index case had normal intellect with a 5- to 10-year history of fatigue and unsteadiness of gait. Her son, however, was severely affected from childhood with progressive gait ataxia, cognitive impairment, bilateral optic atrophy, pigmentary retinopathy, decreased color vision and distal muscle wasting. The index case and her son had a mutation load of 67% and 91%, respectively. Biochemical analysis revealed COX-negative fibers and complete loss of COX II. A second *COX II* missense mutation was identified in a 14-year-old boy with proximal myopathy and lactic acidosis (Rahman et al., 1999). A heteroplasmic T7671A transversion mutation in the N-terminal, membrane-spanning region of COX II resulted in a methionine to lysine amino acid change. Biochemical analysis identified a severe reduction of COX II, COX Vb, COX VIa, COX VIb and COX VIc and a mild reduction of COX I protein. Two other cases of *COX II* mutations have been identified in patients

with a COX deficiency. One example was found in twin brothers with severe lactic acidosis who died after 2 and 12 days, following apnea and bradycardia (Wong et al., 2001). A 8042delAT deletion resulted in a 72 amino acid truncation at the C-terminus. The C-terminal domain of COX II is important for hydrophilic interactions with cytochrome *c*, ligand binding to copper and magnesium ions, as well as for the formation of proton water channels. All of these roles are extremely important for COX function and therefore essential for life. Another G7896A nonsense mutation causes a 123 amino acid truncation of COX II at the C-terminus (Campos et al., 2001). At 3 months of age, the individual presented with psychomotor delay, failure to thrive, hypotonia, hypertrophic cardiomyopathy and pigmentary retinopathy. COX deficiency and mutational heteroplasmy was observed in muscle cells, however only heteroplasmy was observed in blood and fibroblast tissue.

Four *COX III* mutations have been identified and are known to cause COX deficiency. In 1996, a 15 bp microdeletion was identified in a highly conserved region of COX III (Keightley et al., 1996). The 15-year-old female patient presented with muscle cramps and myoglobinuria which lead to the identification of ragged-red fibers with a 64% proportion of COX-negative fibers. The mutant load was 92% in muscle cells and 0.7% in leukocytes. In 1998, a second *COX III* mutation was identified in a 36-year-old woman experiencing lactic acidemia-associated encephalopathy, exercise intolerance and proximal myopathy (Hanna et al., 1998). A G9952A point mutation in the patient's skeletal muscle revealed a loss of the last 13 amino acids of COX III. This mutation appeared to reduce holoenzyme stability and possibly impair COX assembly. A third *COX III* insertion mutation at 9537insC was identified in an 11-year-old female with progressive spastic paraparesis associated with ophthalmoparesis, moderate mental retardation, severe lactic acidosis and Leigh-like lesions in the putamen (Tiranti et al., 2000). The homoplasmic frameshift mutation resulted in undetectable amounts of COX III protein and failure to assemble the holoenzyme by disrupting the formation of smaller subcomplexes. The fourth case of a *COX III* mutation was identified in a patient with mild, childhood onset progressive myopathy with exercise intolerance, lactic acidosis and delayed growth (Horvath et al., 2002). The heteroplasmic G9379A transition mutation

(W58X) in *COX III* resulted in decreased amounts of COX II and COX III in the skeletal muscle.

COX deficiency caused by nDNA mutations

Due to the clinical and genetic heterogeneity of COX deficient patients and the large number of nuclear factors involved in COX biogenesis, the identification of disease-causing mutations has proven difficult. An autosomal recessive inheritance pattern has been observed for many COX deficiencies, leading to the assumption that the mutation lies within the nuclear genome. Although many patients have unknown genetic defects, there are a some that fall within a small group of well-characterized diseases, such as Leigh Syndrome (LS), LS-French Canadian type, fatal infantile COX deficiency and hypertrophic cardiomyopathy and myopathy (Shoubridge, 2001). Interestingly, no genetically characterized COX deficiency has been due to mutations in nuclear-encoded structural subunits of COX. The mutations that have been found are in nuclear-encoded assembly factors required for COX biogenesis. COX assembly factors identified in human disease include SURF1, LRPPRC, SCO1, SCO2, COX10 and COX15. Understanding the role of these assembly factors in COX deficiencies has been highly aided through yeast studies of *pet* mutants deficient in COX assembly.

Leigh Syndrome is an early onset, fatal neurodegenerative disease that presents with bilateral lesions in the brainstem, basal ganglia, thalamus and spinal cord (Shoubridge, 2001). This heterogeneous disease is known to arise from defects in complex I, complex II and pyruvate dehydrogenase, but is most severe when associated with COX deficiencies (Barrientos, 2003). Patients with Leigh syndrome have 10-25% COX activity when compared to wild-type (Shoubridge, 2001). A LS locus was mapped to chromosome 9 by functional complementation of a COX deficiency by the gene, *SURF1*, located on 9q34, which is a homolog to the yeast *pet SHY1* gene. Wild-type *SURF1* cDNA was found to rescue the COX deficiency in LS patient fibroblasts, supporting its role in LS development. Mutational analysis of LS patients has identified mutations in all 9 exons of *SURF1* (Pecina et al., 2004a). The SURF1 protein is a 30 kDa protein that is

embedded in the IMM by two transmembrane domains. *SURF1* mutants arrest COX assembly at subcomplex 2, right before the addition of COX II and III to COX I and COX IV (Figure 1-3). This suggests that SURF1 plays a role in COX II addition during COX assembly (Shoubridge, 2001).

Leigh Syndrome, French-Canadian type (LSFC) is characterized by subacute neurodegeneration of the brain stem and basal ganglia (Mootha et al., 2003). Due to a founder effect, LSFC is unique to the Saguenay-Lac St. Jean region of Quebec with a carrier rate of 1 in 23 individuals and a disease frequency of 1 in 2000 live births. LSFC is distinct from LS because of marked tissue-specificity of the COX deficiency (Shoubridge, 2001). Liver and brain tissues have decreased COX activity, whereas kidney and heart tissues have normal COX activity. Pedigree analysis linked the candidate gene to chromosome 2p21-p16 (Lee et al., 2001), which was later identified as *LRPPRC* (Mootha et al., 2003). Of 22 LSFC patients examined, 21 were found to contain a homozygous A354V mutation. The other patient was a compound heterozygote with an 8 bp deletion in exon 35 that truncated LRPPRC at amino acid 1277. LRPPRC is a leucine-rich pentatricopeptide repeat cassette protein of 130 kDa with 4 potential N-linked glycosylation sites, similar to the ATP-binding site of ATP-dependent kinases (Hou et al., 1994). LRPPRC is a homolog of yeast Pet309p, a yeast protein required for expression of COX I (Xu et al., 2004). Both Pet309p and LRPPRC are thought to function in mRNA-binding due to the presence of PPR repeat motifs. Thus, Pet309p and the human homolog LRPPRC are important for translation and/or stability of COX I mRNA transcripts.

SCO1, SCO2 and COX17 are all copper proteins required for COX assembly in yeast and humans. Both SCO1 and SCO2 have been found to contain mutations that lead to human COX deficiencies (Papadopoulou et al., 1999). Although *COX17* is a potential candidate gene for human COX-deficiency, no *COX17* mutations have been found to lead to human disease (Horvath et al., 2005; Darin et al., 2003; Horvath et al., 2000; Sacconi et al., 2003). Mutations identified in *SCO1* have been observed in patients with hepatopathy and ketoacidotic coma (Valnot et al., 2000a). Two compound heterozygous mutations have been identified: a 2 bp frameshift deletion (363insGA) creating a

truncated protein that lacks the conserved core region of SCO1 and a P174L missense mutation adjacent to the copper-binding motif, CxxxC. The *SCO1* gene spans 17 kb of chromosome 17p13-p12 and contains 6 exons (Petruzzella et al., 1998; Horvath et al., 2000). As mentioned previously, mutations in the yeast homolog cause a loss of Cox2p. Human SCO1 and yeast Sco1p are 40% identical, and both contain a mitochondrial targeting sequence (Valnot et al., 2000a). Human *SCO1* is predominately expressed in respiratory-dependent tissues, including heart, muscle and brain. Several investigations have looked for *SCO1* mutations in COX deficient patients, however few have been identified.

A second SCO locus, *SCO2*, has also been found to contain mutations that lead to human COX deficiency and fatal infantile cardioencephalomyopathy (Papadopoulou et al., 1999). To date, all identified patients are compound heterozygotes and all contain an E140K mutation adjacent to the copper-binding motif, CxxxC. Two cases of an E140K and a Q53X mutation have also been identified. The Q53X mutation truncates the SCO2 protein upstream of the conserved, putative core of the protein. Two other patients have been identified with E140K mutations along with S225F (Papadopoulou et al., 1999) and C133Y (Tarnopolsky et al., 2004), which is located in the copper-binding region. These mutations result in a severe reduction of COX I-III proteins, as well as a mild reduction in COX IV and COX Va. Isolated myoblasts from patients bearing *SCO2* mutations were transfected with *SCO2* by retroviral gene transfer (Jaksch et al., 2001). Post-transfection analysis revealed restoration of COX activity, demonstrating that the *SCO2* mutations are the cause of COX deficiency. Furthermore, the supplementation of copper for patient myoblasts rescued the COX deficiency, suggesting a copper-dependent role for SCO2.

As with copper delivery to COX, heme biosynthesis and placement in the holoenzyme is important for COX function. The heme biosynthetic factors found to contain mutations leading to human COX deficiency include *COX10* and *COX15*. Several COX deficient patients with *COX10* mutations present with leukodystrophy and proximal tubulopathy, (Valnot et al., 2000b) fatal infantile hypertrophic cardiomyopathy and a Leigh-like syndrome (Antonicka et al., 2003a). The COX10 amino acid changes found in these patients include, N204K, D336V, D336G and P225L. These mutations lead to a

mild reduction in all COX subunits, except COX II, which is severely reduced. The encoded *COX10* gene product was first identified in yeast to encode a heme A:farnesyltransferase required for the conversion of protoheme to heme O (Glerum and Tzagoloff, 1994). It is interesting that patients bearing *COX10* mutations maintain normal levels of COX I, since COX I contains both of the heme A sites (Valnot et al., 2000b); (Antonicka et al., 2003a). Heme A does not appear to stabilize COX I but rather induce a conformational change within COX I that confers binding of subsequent COX subunits. As with mutations in *COX10*, mutations in *COX15* have been found to cause fatal infantile hypertrophic cardiomyopathy. Three patients have been identified with a R217W mutation, of which two are compound heterozygotes (Antonicka et al., 2003b) and one is homozygous (Oquendo et al., 2004). In both compound heterozygous cases, the mutation results in missplicing and truncation of *COX15* mRNA, which leads to mRNA instability and a functionally null allele (Antonicka et al., 2003b). Along with COX deficiency, one patient showed a reduction of heme A and slight accumulation of heme O. The homozygous R217W mutation in *COX15* was found in a patient with classical Leigh syndrome (Oquendo et al., 2004). The clinical heterogeneity observed in the patient containing a R217W *COX15* mutation highlights the difficulties in understanding and identifying mutations associated with COX deficiency.

COX biogenesis is a complex process involving the coordination of both the nuclear and mitochondrial genomes in order to assemble in the inner mitochondrial membrane. In addition to the polypeptides of the complex itself, there are a host of assembly proteins responsible for the synthesis and placement of the prosthetic groups. We are only beginning to understand the processes involved in producing a functional COX holoenzyme, and the consequences that result from mutations in the mitochondrial-encoded subunits and assembly factors. Mutations have been identified in the catalytic subunits COX I, COX II and COX III and in the assembly factors SCO1, SCO2, SURF1, LRPPRC, COX10 and COX15 and are associated with human COX deficiencies. However, a large number of COX deficient patients have an unknown underlying molecular basis. Continued investigations of COX assembly will offer insight into the molecular and pathogenic mechanisms that underlie human COX deficiencies and will

likely involve the identification of additional COX assembly factors. This thesis will illustrate my attempt at identifying additional gene-products involved in COX biogenesis. As well, this thesis describes the characterization of human COX17P and addresses the possibility that COX17P provides functional redundancy in the human COX assembly pathway, thus providing an explanation as to why no *COX17* mutations have been identified in human disease.

Chapter 2 ❖ Materials and Methods

Strains and Media: Bacteria and Yeast

E. coli were grown in liquid LB media (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.1% glucose) and after transformation were plated on LB (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.1% glucose, 2% agar) containing 50 µg/ml of ampicillin (Sigma) for solid media.

The yeast strains used in this study are summarized in Table 2-1. The yeast *cox17* knockout strain was created by insertion of a *TRP1* fragment into the *Eco*RI site within the *COX17* coding region (Glerum et al., 1996a), which disrupts expression of a functional Cox17 protein. Yeast were plated on YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and stored at 4ºC. For yeast transformations, yeast were cultured in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose), a medium rich in glucose. After transformation, yeast were plated on minimal media (WO – 0.67% nitrogen base without amino acids, 2% glucose, 2% agar) supplemented with the appropriate requirements (0.5µg uracil, 0.5µg adenine, 0.5µg histidine, 0.5µg tryptophan, 0.5µg leucine). To examine respiratory ability, yeast were plated on ethanol/glycerol (EG) (1% yeast extract, 1% peptone, 2% ethanol, 2% glycerol, 2% agar), both of which are non-fermentable carbon sources. For mitochondrial isolation, yeast were grown in liquid galactose (GAL) media (1% yeast extract, 1% peptone, 2% GAL) to maximize mitochondrial biogenesis.

DNA clones and constructs

Yeast

Construction of SCO2/ST6

In order to create pSCO2/ST6, a 1.9kb *Sac*I-*Sph*I fragment containing *SCO2* from pSG74/ST4 (in YEp352, containing a *Sma*I fragment of *SCO2*) was inserted into YEp351 (Hill et al., 1986). YEp351 is a yeast episomal plasmid containing a leucine selective marker for selection in yeast, an ampicillin selective marker for selection in *E. coli* and a 2µ marker for high copy expression.

Pre-existing DNA Clones

pSCO1/ST10 – This is a YEp351 plasmid containing a 1.9kb *Eco*RI *SCO1* insert treated with Klenow and inserted into the *Sma*I site of YEp351.
pG74/ST21 – This YEp351 plasmid contains a 750 bp *Hind*III fragment containing the *COX17* coding region.

Human

Construction of pCDNA3.1-hCOX17NFL (COX17-FLAG) and pCDNA3.1-hCOX17NFLP (COX17P-FLAG)

To create pCDNA3.1-hCOX17NFL, the human *COX17* cDNA was amplified from pG74H/T1, using primers 17HFLAGN-F (5'-gacgacgatatcaccatgggtggtgccgactacaaggacgatgacgataagatgccgggtctggttgactc-3') and 17HFLAGN-R (5'-gacgacctcgagccatttcatattttaaatcctag-3') (made by Punter, 2003). This

primer pair introduced an *Eco*RV site upstream of the *COX17* coding region, an in-frame FLAG epitope at the 5' end of the *COX17* coding region, and an *Xho*I site downstream of the *COX17* coding region. The *COX17* cDNA fragments containing the added restriction sites and FLAG epitope were ligated into the pCDNA3.1/hygro+ vector (Invitrogen Life Technologies). I created the vector pCDNA3.1-hCOX17NFLP by the same means as pCDNA3.1-hCOX17NFL, but amplified *COX17P* from pMGL4-hCOX17P (see below). The sequences of the plasmid inserts were verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor).

Construction of pMGL4-hCOX17P and pMGL4-hCOX17

The human *COX17* pseudogene was amplified from human genomic DNA using primers COX17H-4 (5'-agttcgtcaaagaactccca-3') and COX17B-2 (5'-ttaaagaaatcggacgaattgg-3') and cloned into the pGEM T-Easy vector (Promega) generating pGEM-hCOX17P. After digestion with *Eco*RI and Klenow fragment to blunt the digested ends, the *COX17* pseudogene cDNA fragment was ligated into the multiple cloning site of *Sma*I-digested pMGL4, creating construct pMGL4-hCOX17P.

Expression of pMGL4-hCOX17 and pMGL4-hCOX17P is driven by the *S. cerevisiae ADH1* promoter. To allow constitutive expression of human *COX17* in yeast, the human *COX17* cDNA was amplified from pG74H/T1 (Amaravadi et al., 1997) and cloned into the pGEM T-Easy vector (Promega), generating pGEM-hCOX17 (Punter, 2003). After linearizing the *COX17* cDNA fragment with *Sph*I and treatment with Klenow, the vector was digested with *Sac*I and ligated into the multiple cloning site of *Sac*I - *Sma*I digested pMGL4 (D.M. Glerum, unpublished), creating construct pMGL4-hCOX17 (Punter, 2003).

Molecular Biology techniques

Transformation of competent *E.coli* cells

E. coli cells are made competent following the protocol of Inoue et al (Inoue et al 1990) and stored in 200µl aliquots at -80ºC. Aliquots were thawed at room temperature for 20 minutes and placed on ice. Cells were incubated for 30 minutes on ice after the addition of 10µl of plasmid DNA. After incubation, the cells were heat shocked at 42ºC for 30 seconds and placed on ice again. After the addition of 800µl of LB media, the cells were incubated at 37ºC for 1 hour, shaking at 225 rpm. The cells were plated on ampicillin-selective LB plates and grown overnight at 37ºC.

E. coli Plasmid Miniprep

Individual transformed *E. coli* cells were patched on selective LB agar plates and incubated overnight at 37ºC. A scraped area (~1cm^2) of *E. coli* was mixed with 1ml of miniprep buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme, 0.02 mg/ml RNase A) and incubated on ice for 1 minute. After incubation, 200 µl of 0.2 M NaOH, 1% SDS was added, followed by 150 µl of 7.5 M ammonium acetate (NH_4OA_C). The sample was centrifuged at 14,000 rpm for 8 minutes and the supernatant

Table 2-1 : *Summary of yeast strains*

Yeast Strains	**Genotype**	**Amino Acid Changes**
W303ΔCOX17	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1*	
α W303ΔCOX17/pCEN/17RM229	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17RM229*	C57Y
a W303ΔCOX17/pCEN/17RM235	a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17RM235*	D34V
α W303ΔCOX17/pCEN/17RM409	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17RM409*	C26R
α W303ΔCOX17/pCEN/R33D	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17R33D*	R33D
α W303ΔCOX17/pCEN/D34R	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17D34R*	D34R
α W303ΔCOX17/pCEN/17RM418	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17RM418*	C47R
α W303ΔCOX17/pCEN/17RM577	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1 + pCEN/17RM577*	C24R

collected and added to 300 µl of isopropanol containing 0.2% Triton X-100. The sample was centrifuged again for 8 minutes. The final plasmid DNA pellet was washed once with 80% ethanol, 0.2 mM EDTA and once with 70% ethanol. The dried pellet was dissolved in 30 µl deionized, distilled water (ddH_2O).

Purification of DNA Fragments with Glass Milk

DNA was electophoresed in 1 – 2.5% agarose gels and the DNA fragment of interest was excised from the gel and weighed. Three volumes of NaI (6 M NaI, 12 mM Na_2SO_3) was added to gel slice (ie. slice = 0.15 g, add 450 µl of NaI) which was melted by heating at 55-65°C for approximately 10 minutes. 10 µl of glassmilk (made with crushed silica) was added to the sample, which was incubated for a total of 10 minutes and was vortexed every two minutes. The sample was pelleted at 14,000 rpm for 20 seconds and washed three times with 700 µl of NEET solution (100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.5, 50% ethanol). The pellet was mixed with 10 µl of ddH_2O and incubated at 55-65°C for 5 minutes. The sample was centrifuged for 30 seconds and the supernatant, containing the DNA, was collected. The pellet was once again incubated with water, centrifuged and the supernatant collected. The collected supernatants were spun down for two minutes at 14,000 rpm to remove any residual glassmilk. The final sample was analyzed by electrophoresis.

Triton Plasmid DNA Preparation

Transformed *E. coli* cells were incubated on ice for 30 minutes with 1 ml of sucrose buffer (5% sucrose, 50 mM Tris pH 8.0) and 1 ml of lysozyme buffer (25 mM Tris pH 8.0, 125 mM EDTA, 0.04 mg/ml ribonuclease A and 5 mg/ml lysozyme). One milliliter of lysis buffer (0.3% Triton X-100, 185 mM EDTA pH 8.0, 150 mM Tris pH 8.0) was also added to the *E. coli* cells. Cells were spun at 4°C in a Beckman thick-walled ultracentrifuge tube at 40,000 rpm for 20 minutes. The supernatant was collected and added to an equal volume of water-saturated phenol. The cells were centrifuged at 4,000 rpm for 5 minutes at 4°C. The upper aqueous phase was washed 3-4 times with ether, followed by the addition of 0.05 volumes of 5 M NaCl and 3 volumes of 100% ethanol. The sample was spun at 4,000 rpm for 10 minutes at 4°C to form a small, oily DNA pellet. The pellet was dissolved in 2 ml of 2 M NH_4OAc and precipitated with 3 volumes of 100% ethanol, spun down and repeated. The final DNA pellet was rinsed once with 80% ethanol, 0.2 mM EDTA and once with 70% ethanol and dissolved in an appropriate volume of deionized, distilled water (ddH_20).

Yeast Transformation by Lithium Acetate Method

This protocol has been modified from the method described by Schiestl and Gietz (Schiestl and Gietz, 1989). Yeast colonies were inoculated in 10 ml of YPD and grown overnight at 30°C, shaking at 225 rpm. 2 ml of overnight culture was innoculated into 75 ml of fresh YPD and grown for an additional 3-4 hours at 30°C, shaking at 225 rpm. Cells were pelleted at 3,000 rpm for 5 minutes and washed in 10 ml of TEL (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1 M LiAc). The washed pellet was resuspended in 450 µl of TEL and 100 µl aliquots were distributed in 1.5 ml Eppendorf tubes. Salmon sperm DNA (10

mg/ml) was denatured at 90°C for 10 minutes before adding 5 µl to each aliquot of cells. A 5 µl sample of DNA to be transformed was also added to each aliquot and incubated at room temperature for 30 minutes without shaking. 700 µl of PEG/TEL buffer (40% polyethylene glycol, 10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1 M LiAc) was mixed into each aliquot and incubated an additional 45 minutes at room temperature. The cells were heat shocked at 45°C for 13 minutes and centrifuged for 10 seconds at 14,000 rpm. The cells were washed twice with 200 µl of TE (10 mM Tris-Cl pH 7.5, 1 mM EDTA) and plated on selective minimal media (WO) plates and grown at 30°C for three nights.

Transfection of COS-7 cells

For subcellular fractionation experiments, COS-7 cells (obtained from Dr. Michael Walter's laboratory) were plated at a density of 2.25×10^6 cells per 150 mm tissue culture plate, 24 hours prior to transfection. Cells were transfected with 7.5 µg of pCDNA3.1-hCOX17NFL or pCDNA3.1-hCOX17NFLP using the FuGENE 6 transfection reagent (Roche). Forty-eight hours after transfection three 150 mm plates of transfected or untransfected COS-7 cells were harvested for subcellular fractionation.

Yeast Revertants

The cox17 mutant strains, D34R and C26R were inoculated in 10 ml of YPD liquid media and grown overnight at 30°C. The cells were spun down for 5 minutes at 3000 rpm and plated on EG plates and grown at 30°C for 5 nights. Single colony revertants were streaked for singles on YPD plates and grown for 2 nights at 30°C. The YPD plates were replica plated to EG and WO plates with three different amino acid mixes: 1) histidine, leucine, adenine, 2) histidine, leucine, adenine, tryptophan, and 3) histidine, uracil, adenine, and leucine. Revertant colonies that grew on all three of these plates were selected and scored for growth on EG. The revertant strains were stored on YPD plates at 4°C and frozen in glycerol media at 80°C for further use.

Yeast Colony Polymerase Chain Reaction

Yeast reversion strains were patched (1 cm^2) on YPD and grown overnight (~10-15 hours) at 30°C. The yeast patches were scraped and mixed into 100 µl of ddH_2O and boiled for 10-15 minutes. The boiled cells were spun at 14,000 rpm for 15 seconds to pellet the cells. 5 µl of the supernatant was used in a 35 µl PCR reaction. Three products were independently amplified by PCR. The *SCO1* gene was amplified using primers SCO1A (5'-cttctgagtcacgaaatcaacagta-3') and SCO1D (5'-atacagcgtcatgtctaccacacta-3') to create a 1.5 kb product. The mutant *cox17* sequence located on the YEp351 centromeric plasmid was amplified using primers 351-1 (5'-gagcggataacaatttcacac-3') and 351-2 (5'-ggttttcccagtcacgacg-3') to create a 573 bp product. The SCO2 gene was amplified using primers SCO2-F (5'-cttgatcctttcgctcttttag-3') and SCO2-B (5'-tcccgcatgtttacgccctt-3') to create a 1.2 kb product. The PCR samples were incubated for one minute at 94°C and were held at 80°C for the addition of three units of Taq (Invitrogen). The samples were heated at 55°C for 1 minute, 72°C for 1 minute and at 94°C for 30 seconds. The primer annealing, extension and product denaturing steps were repeated 34 times followed by heating at 55°C for 1 minute and 72°C for 5 minutes. The PCR samples were subjected to gel electrophoresis in 1 – 2.5% agarose gels. After gel

purification, the DNA samples were sequenced using the ABI PRISM ® 3100-*Avante* Genetic Analyzer (Applied Biosystems Inc.).

Cells and Culture

All cells were cultured in a Nuaire CO_2 Water-Jacketed incubator at 37°C with 5% CO_2 in T_{75} flasks (Corning). HUH7 and HeLa cells were grown in DMEM (Dulbecco's Modified Eagle's Media) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of antibiotic/antimycotic (Gibco, 15240-062). Jurkat cells were grown in RPMI 1640 supplemented with 25 mM Hepes (pH 7.4), 10% FBC and 100 µM β-mercaptoethanol. Raji and THP-1 cells were grown in RHFM media supplemented with 10% FBS and 100 U/ml of antibiotic/antimycotic (Gibco, 15240-062). NK92(DK) cells were grown in RHFM media supplemented with 12% FBS and 100 U/ml of antibiotic/antimycotic (Gibco, 15240-062). The remaining cells, hCD8, RPMI 8866, YTS and 221 were obtained directly from Dr. Chris Bleakley's laboratory (Department of Biochemistry, University of Alberta).

RNA Preparation

Total cellular RNA was isolated using the RNeasy Mini Kit (QIAGEN, 74104) following 'RNeasy Mini Protocol for Isolation of Total RNA from Animal Cells.' The cells were homogenized by passing lysate 10 times through a 22-gauge needle. RNA from each cell line was isolated from 7 million cells.

Reverse Transcriptase-Polymerase Chain Reaction

Reverse transcription was performed on liver, spleen, heart muscle, skeletal muscle, brain, lung, thymus (kindly provided by Dr. Heather McDermid) and retinal (kindly provided by Dr. Michael Walter) total cellular RNA. Total cellular RNA of HepG2 cells were provided by Dr. Diane Cox. The remaining RNA samples were isolated following the RNeasy Mini Kit as described above. Total cellular RNA was subjected to reverse transcription using Oligo-dT as a primer for first strand synthesis to create human *COX17* cDNA and using HCOX17HP-R (5'-gggctctcatgcattccttgtgg-3') as a primer for first strand synthesis to create human *COX17* pseudogene cDNA. Total RNA (~1 µl, unknown concentration) was incubated with 10 units of RNase-free DNaseI (Roche) in a total volume of 11 µl with DEPC-H_2O for 45 minutes at 37°C, and 10 minutes at 70°C. Oligo-dT (0.5 µg) or 5 pmol of HCOX17HP-R were incubated with the sample for 5 minutes at 70°C and held at 4°C. To the sample, 4 µl of first-strand buffer (Invitrogen), 10 mM dNTPs (Invitrogen), 0.1 M dTT (Invitrogen) and Superscript II (Invitrogen) were added. Superscript II was only added to the water control (c) and positive control (+), but not to the negative control (-). The sample was incubated for 45 minutes at 42°C and 5 minutes at 95°C. Once completed, the RT samples were stored at -20°C.

Human *COX17* cDNA was amplified using the primers HCOX17HP-R and HCOX17H-F (5'-cggaagtgactgcggacgaat-3'). The positive, negative and control samples were subjected to PCR. The samples were incubated for one minute at 94°C and were held at 80°C for the addition of three units of Taq (Invitrogen). The samples were heated at 54°C for 20 seconds, 72°C for 20 seconds and at 94°C for 15 seconds. These primer

annealing, extention and product denaturing steps were repeated 34 times followed by heating at 54°C for 20 seconds and 72°C for 5 minutes. The PCR samples were subjected to gel electrophoresis in 1 – 2.5% agarose gels. PCR products of amplified genomic COX17P were cloned into a pGEM T-Easy vector (Promega) and sequenced using an ABI PRISM® 3100-*Avante* Genetic Analyzer (Applied Biosystems Inc.) for sequence confirmation. Human *PAX6* was amplified using the primers PAX6-10F (5'-gtagacacagtgctaacctg-3') and PAX6-10R (5'-cccggagcaaacaggtttaa-3') to ensure that there was no genomic contamination in the cDNA samples. As a positive control *PAX6* was also amplified from genomic DNA (25 ng). The same PCR protocol used for cDNA amplification was used for *PAX6* amplification, except the primer annealing temperature was 60 °C.

Biochemical Techniques

Preparation of Intact Yeast Mitochondria by Zymolyase

Yeast were shaken at 30°C (225 rpm) in 10 ml of GAL medium overnight. A 2 ml aliquot of the overnight culture was inoculated in 100 ml of GAL and was shaken overnight at 30°C. This overnight culture was split three times into 800 ml of GAL medium and shaken overnight at 30°C. The final overnight culture was spun at 2,000 rpm for 7 minutes. The cell pellet was resuspended in 1.2 M sorbitol and centrifuged at 4°C at 6,000 rpm for 10 minutes. The cell pellet was weighed and 3 ml/g of cells of digestion buffer (1.2 M sorbitol, 75 mM NaP_i pH 7.5, 1 mM EDTA, 0.01% β-mercaptoethanol, 0.45 mg/ml Zymolyase) was added. Yeast was incubated at 37°C for approximately 1.5 hours for respiratory deficient strains and 2-3 hours for respiratory competent strains. The extent of lysis was assessed under a light microscope. 10 µl of cells were added to 10 µl of ddH_2O on a microscope slide for analysis. After ensuring adequate lysis, the cells were washed four times with 1.2 M sorbitol following centrifugation at 6,000 rpm for 10 minutes after each wash. The spheroplasts were resuspended in STE buffer (0.5 M sorbitol, 20 mM Tris pH 7.5, 0.5 M EDTA) and homogenized in a Waring blender for 20 seconds. 5 µl PMSF (20 mM) was added before the spheroplasts were spun at 2,500 rpm for 10 minutes. The supernatant was collected and spun again to remove remaining debris. To sediment the mitochondria, the supernatant was spun at 12-15,000 rpm for 15 minutes. A collection of 2-4 ml of supernatant was stored as the post-mitochondrial supernatant or cytosolic fraction. The mitochondrial pellet was washed three times with STE buffer following centrifugation at 12,000 rpm for 10 minutes. Mitochondria were resuspended in 20 mM Tris and 0.4 mM PMSF.

Determination of Protein by Folin Procedure

Protein concentrations were determined using the method of Lowry et al. (Lowry et al., 1951). In 13 x 100 mm test tubes, 5 µl of isolated mitochondria and 10 µl of post mitochondrial supernatant were added to water to a total volume of 600 µl. A 600 µl water blank was also prepared. 3 ml of copper reagent (0.01% $CuSO_4$, 0.02% NaK tartrate, 1.96% Na_2CO_3 in 0.1 N NaOH) are incubated with each sample for 10 minutes. To each sample, 0.3 ml of Folin reagent (1:1 dilution of stock, Sigma) was added and

mixed immediately. Each sample was heated to 90°C for 2 minutes and re-equilibrated to room temperature before analysis. The absorbance was read at 750 nm against the reagent blank using a Shimadzu UV-Visible UV-1601 PC spectrophotometer.

Isolation of mitochondria from COS-7 cells

Each plate of COS-7 cells (obtained from Dr. Michael Walter's laboratory) was washed with sucrose buffer (0.25M sucrose, 20mM Tris, 1mM EDTA, pH 7.4). After removal, an additional 2 ml of sucrose buffer was added and the cells were scraped off the plate using a rubber policeman. After each plate was scraped, the suspension was transferred to the next plate in order to minimize the total amount of buffer the cells are suspended in. Once all the cells were collected they were transferred into a chilled Teflon homogenizer and homogenized (30 strokes). The cells were transferred to a new tube and centrifuged for 7 minutes at 3000 rpm (4°C). The supernatant was kept and centrifuged again for 7 minutes at 3000 rpm (4°C). After the second spin the supernatant was collected again and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant from this spin was the post-mitochondrial supernatant. Several milliliters were stored for further analysis. The mitochondrial pellet was resuspended in a small volume of sucrose buffer and spun again at 10,000 rpm for 10 minutes at 4°C. The final pellet was resuspended in 0.5 ml of sucrose buffer and transferred into a 1.5 ml Eppendorf tube and centrifuged at 13,000 rpm for 10 minutes at 4°C. The pellet was resuspended in a small amount (100 – 400 µl) of sucrose buffer and stored at -80°C.

Immunofluorescence

COS-7 cells were plated on glass coverslips at a density of 2 x10^5 cells per 35 mm tissue culture plate, 24 hours prior to transfection. Cells were transfected with pCDNA3.1-hCOX17NFL or pCDNA3.1-hCOX17NFLP using the FuGENE 6 transfection reagent (Roche) described above. Forty-eight hours after transfection, COS-7 cells were stained with MitoTracker Red CMXRos (Molecular Probes) at a concentration of 30 nM. After a 20 minute incubation at 37°C the cells were washed 2 times in PBS (140 mM NaCl, 10 mM NaP_i pH 7.5). Cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 in PBS. Immunostaining was carried out using mouse monoclonal α-FLAG M2 antibody (F-3165, Sigma) or mouse monoclonal anti-cytochrome c antibody (BD PharMingen), followed by anti-mouse IgG FITC-conjugated secondary antibody (Jackson ImmunoResearch). Glass coverslips were mounted on glass slides using the ProLong Antifade kit (Molecular Probes) and the immunofluorescence visualized using a LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100M microscope. Cells transfected with pCDNA3.1-hCOX17NFL or pCDNA3.1-hCOX17NFLP were visualized by excitation at 488 nm, with the fluorescence signal collected using a 505-530 nm band pass filter. The MitoTracker Red signal was induced by excitation at 543 nm, with the fluorescence signal collected using a 560 nm long pass filter.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western Blotting

The protein samples were prepared in sample buffer (4x stock: 0.19M Tris-HCl pH 6.8, 3.85% SDS, 0.04% β-mercaptoethanol, 38.5% glycerol, 0.02-0.05% bromophenol blue w/v). The samples were loaded onto a 12% (Laemmli 1970) or 15% with glycerol (Glerum and Tzagoloff 1998) SDS (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel and separated at 150V for 1:15 hours in running buffer (25 mM Tris, 191 mM glycine, 8.7 mM SDS). The gel was transferred onto nitrocellulose (Trans-Blot Transfer Medium, BIO-RAD) using the Mini-Protean II system (BIO-RAD) in cold transfer buffer (200 mM glycine, 25 mM Tris, 20% methanol) at 100V for 30 minutes. Following transfer, the nitrocellulose membrane was stained with Ponceau S (2% Ponceau S (Sigma), 30% trichloroacetic acid, and 30% sulfosalicylic acid) to mark the sample lanes and ladder (low molecular weight ladder). The Ponceau was rinsed off the nitrocellulose membrane with water and blocked for 1 hour in blocking buffer (3% milk in rinse buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100)). Primary antibodies were added to the blocking buffer as follows: mouse monoclonal α-FLAG M2 antibody (F-3165, Sigma) 1:1000, mouse monoclonal anti-human farnesyl transferase antibody (gift of Dr. Ian MacDonald-(MacDonald et al., 1998)) at 1:500, mouse monoclonal anti-human COXI antibody (A-6403, Molecular Probes) at 1:2000, and rocked overnight at 4°C. After overnight incubation with the primary antibody, the nitrocellulose membrane was washed 3 times for 5 minutes in rinse buffer plus salt (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1 M NaCl, 0.1% Triton X-100). The blot was placed on a rocking platform with secondary antibody (goat anti-mouse secondary antibody (#554002, BD Transduction Laboratories) for 1 hour at room temperature, followed by three 5-minute washes with rinse buffer plus salt. Protein detection was carried out using a 1:1 mix of chemiluminescense solution A (98.6 mM Tris pH 8.0, 0.0043% Coumaric Acid, 0.0099% Luminol) and chemiluminescense solution B (100 mM Tris pH 8.0, 0.018% hydrogen peroxide) and exposed to Kodak X-OMAT film and developed using a M35A X-OMAT processor (Kodak).

Chapter 3 ❖ Results

MITOCHONDRIAL COPPER TRANSPORT STUDIES IN YEAST

Suppression of cox17 point mutants

The delivery of copper to COX is an essential step in COX assembly and is required to maintain respiratory competent cells. The current model of copper delivery to COX involves the copper-binding proteins Cox17p (Glerum et al., 1996a), Sco1p (Glerum et al., 1996b) and Cox11p (Carr et al., 2002). Cox17p is thought to deliver copper to Sco1p (and possibly Sco2p) and Cox11p, which subsequently transfer copper to the Cu_A and Cu_B sites in COX, respectively. To further understand this copper transport pathway, I investigated the ability of Sco1p and Sco2p to suppress several respiratory deficient *cox17* point mutants and a null *cox17* strain. Our lab has generated over 60 *cox17* point mutants by a combination of site-directed and random mutagenesis (Punter, 2003). Of these, only 12 yielded a respiratory deficient phenotype when transformed into a *cox17* knockout strain at low-copy number (Punter, 2003). From this collection, I chose to study several mutants incapable of utilizing the non-fermentable carbon source, ethanol/glycerol (EG). The *cox17* mutants that I investigated can be broken into four groups: *cox17* knockout mutant, *cox17* copper-binding mutants, *cox17* import mutants (incapable of importing into mitochondria), and other *cox17* point mutants (unknown function).

We are working with complex IV deficient yeast strains and are using EG medium as an indicator of COX function. When a yeast strain grows on EG, it suggests that COX has correctly assembled and is functional. A lack of growth on EG indicates a misassembled and non-functional COX complex. The mutant strains C24R, C26R, C47R, C57Y, R33D and D34R are completely respiration deficient, whereas the mutant strain, D34V, becomes respiratory competent at 1/4 of the rate of wild-type yeast strains (Figure 1-7). Two of these mutants, C47R and C57Y have no detectable Cox17p in the mitochondria by Western blot analysis and are therefore referred to as the *cox17* import mutants. These residues are now known to be required for mitochondrial import via the

disulphide relay system (Chacinska et al., 2004). The remaining mutants have reduced levels of Cox17p present in both the cytosol and mitochondria. The C24R and C26R alleles contain mutations in the copper-binding domain, KPCCxC and are likely incapable of binding copper. These mutants are referred to as the *cox17* copper-binding mutants.

To further characterize the *cox17* mutants, I transformed the *cox17* alleles C24R, C47R, C57Y, D34V, C26R, R33D and D34R with high-copy plasmids containing *SCO1* and *SCO2* and examined their ability to suppress a lack of growth on EG (Table 3-1). These alleles were also transformed with wild-type *COX17* as a control and, as expected, all strains grew on EG when expressing Cox17p. Sco1p was able to restore growth in all of the *cox17* mutants at half the rate of wild-type strains, except D34V, which was rescued at wild-type level, and αΔCOX17, which was rescued at 1/4 the rate of wild-type strains. The ability of Sco1p to suppress *cox17* mutants and the *cox17* knockout suggests that Sco1p receives copper from another source. Sco2p did not act as a high copy suppressor of the C26R, R33D or αΔCOX17 strains, however, it was found to rescue C24R, D34R, C47R and C57Y at 1/4 the rate of wild-type and D34V at 1/2 the rate of wild-type. These results suggest that Sco2p may interact with the Cox17p residues, C26 and R33.

Table 3-1 : *High-copy suppression of cox17 mutants by Sco1p, Sco2p*

Growth rate on the non-fermentable carbon source, ethanol/glycerol. After transformation and prototrophic selection, transformants were replica plated to EG and grown at 30°C. All mutants were completely respiratory deficient on EG (growth rate = 0), except D34V which grew on EG at one quarter the rate of wild-type strains (1/4). Growth rate = scoring of growth of *cox17* mutants, as judged by comparison to the growth of a wild-type yeast strain. Sco1p = growth rate of mutants transformed with high-copy plasmid, pSCO1/ST10, containing *SCO1*; Sco2p = growth rate of mutants transformed with high-copy plasmid, pSCO2/ST6, containing *SCO2*; Cox17p = growth rate of mutants transformed with high-copy plasmid, pG74/ST21, containing *COX17*.

***cox17* mutants**	**Growth Rate**	**Cox17p**	**Sco1p**	**Sco2p**
C24R	0	1	1/2	1/4
C26R	0	1	1/2	0
R33D	0	1	1/2	0
D34R	0	1	1/2	1/4
D34V	1/4	1	1	1/2
C47R	0	1	1/2	1/4
C57Y	0	1	1/2	1/4
αΔCOX17	0	1	1/4	0

Spontaneous Reversion in cox17 mutants

Although the exact mechanism of copper transport to COX has not been delineated, it is currently thought to involve three proteins. The results of my previous experiment suggest that other assembly factors or chaperones are involved in copper transport to COX and have yet to be identified or defined. A multicopy suppressor screen of *cox17* mutants may help to identify new gene products involved in COX assembly. Yeast genetic screens are extremely useful in identifying other gene products involved in a specific cellular pathway or in a parallel pathway.

Our lab has a yeast genomic library, L13, which was created from the haploid yeast strain, D273. This genomic library was used for a multicopy suppressor screen in the "unknown" *cox17* mutant, R33D and the *cox17* copper-binding mutant, C26R. Yeast genomic libraries are created by enzymatically digesting nuclear DNA into 2-15 kb fragments, which are subsequently ligated into an expression vector. Because the genome is broken into fragments it would be ideal to have three times the number of transformants as there are genes in the yeast genome. Since yeast have approximately 6,000 genes, a successful multicopy suppressor screen would have 18,000 transformants. I transformed R33D and C26R with 75 μg of the L13 library on three independent occasions and obtained a maximum of 6,300 transformants. Transformants were plated on minimal media supplemented with histidine and adenine (WO+HA). The remaining auxotrophies were not required on the WO plates since the L13 library was created in a *LEU2* vector, the *cox17* R33D and C26R mutants were knocked out with *TRP1* and the mutant *cox17* alleles were on a *URA3* plasmid (Table 2-1). The transformants were then replica plated to EG and scored for growth. I noticed several colonies that were not growing on the WO+HA plates, but were growing on EG plates. To investigate these colonies further, I streaked several colonies from the EG plates on YPD plates and replica plated them onto minimal media supplemented with different combinations of adenine, leucine, tryptophan, histidine and uracil. I observed many colonies that could not grow in the absence of uracil or tryptophan, which indicated either a loss of the *URA3* plasmid, or a loss of *TRP1* at the knockout site. The low transformation efficiency and the presence of revertants lead us to abort the use of the L13 library for a multicopy suppressor screen.

Disadvantages of the L13 library included: 1) the library was created from a wild-type yeast strain, thus containing the known suppressors *SCO1* and *SCO2*, as well as the complementing gene, *COX17* and 2) there appeared to be a high reversion rate of yeast transformants plated on EG. In order to eliminate the known suppressors and the complementing *COX17* gene from a genomic library, I generated a yeast *SCO1/SCO2/COX17* triple knockout strain by mating a double *COX17::TRP1/ SCO1::URA3* strain to a *SCO2::HIS3* strain. After prototrophic selection to ensure the generation of a triple knockout, the yeast colonies were replica-plated to EG and their growth rates observed. The triple *COX17/SCO1/SCO2* strain was completely respiratory deficient as determined by lack of growth on EG. To generate a genomic library of this strain, I isolated nuclear DNA and partially digested it (500 µg) with *Sau*3A, which resulted in 2-15 kb fragments of DNA. The fragments were gel-purified and ligated into a high copy yeast episomal plasmid (YEp 351/Kan) with Ampicillin and Kanamycin markers for selection in *E. coli*. However, following several ligation reactions with varying amounts of digested nuclear DNA and YEp 351/Kan, I observed poor transformation efficiencies. In many of the transformation experiments, there were more colonies on the control plate (underwent transformation process without any transforming DNA) than on the plate of transformed cells. Although many attempts were made, I could not successfully create a genomic library of the *COX17/SCO1/SCO2* null strain. Once this genomic library has been completed, it will be very useful for a multicopy suppressor screen of various *cox17* mutants.

To determine if the *cox17* mutants reverted because of the transformation process, I plated overnight cultures (unknown concentration) of the stock strains D34R, C26R, R33D and C57Y on EG and incubated them at 30°C. These mutants are known to be completely respiratory deficient on EG, however when plated at high density, several colonies had gained respiratory competency. These revertant colonies grew on EG, anywhere from one night to 6 nights, and when plated on selective media, did not segregate with the correct auxotrophies. The *cox17* mutants were knocked out with *TRP1* at the *COX17* locus and contained mutant *cox17* on a *URA3* plasmid. We would normally expect these W303 mutants to require histidine, adenine and leucine for growth on WO

plates. (Table 2-1). However, some of the revertants I observed could grow in the absence of histidine, adenine or leucine and grew on EG, whereas others required all three auxotrophies. The revertant colonies that grew in the absence of histidine, adenine or leucine were discarded. I selected 14 D34R and C26R spontaneous revertant colonies that displayed all three auxotrophies on WO plates and that were respiratory competent as determined by growth on EG (Table 3-2). The revertants, D34Rr1-6, grew on EG at rates comparable to wild-type strains, whereas the revertants, D34Rr7-9, grew on EG at $1/6^{th}$ the rate of wild-type strains. The revertant, C26Rr2, grew on EG at half the rate of wild-type strains and C26Rr3-6 grew on EG at 1/4 the rate of wild-type strains. These revertants had become respiratory competent, likely due to an extragenic mutation in a gene essential for COX function. To eliminate the candidate copper-binding proteins as sites for the extragenic mutation, the *SCO1* and *SCO2* genes were screened for mutations. I also examined the *COX17* gene, located on the *URA3* plasmid, to ensure the correct mutant *cox17* allele was still present and that there were no additional mutations. Each gene sequence was amplified by yeast colony PCR, gel-purified and sequenced. No mutations were identified in the *SCO1* and *SCO2* genes, and the correct *cox17* mutation was identified in all of the revertant colonies. Interestingly, when I sequenced *cox17* from five of the D34R revertants (r1-5), I identified a portion of the *OST1* gene (Figure 3-1). Using *URA3*-plasmid primers, the first 578 bp of the coding region of *OST1* and 82 bp upstream of the methionine start site were identified in the sequence. *OST1* encodes the alpha subunit of the oligosaccharyltransferase complex located in the lumen of the endoplasmic reticulum, which catalyzes asparagine-linked glycosylation of newly synthesized proteins. The *URA3*-plasmid primer sequences were BLAST against the *Saccharomyces* Genome Database (http://www.yeastgenome.org), which revealed no sequence matches. As an alternate method of amplifying the *cox17* mutant, I attempted to isolate the *URA3* plasmid before amplifying *COX17* by PCR. However, I failed to isolate the *cox17* plasmid, indicating the plasmid may have integrated into the genome. To determine if this was the case, I used *COX17*-specific primers for yeast colony PCR and successfully amplified and sequenced the mutant *cox17* allele.

Table 3-2 : *Growth rate of revertant strains*

Revertant Strain	Growth Rate
D34Rr1	1
D34Rr2	1
D34Rr3	1
D34Rr4	1
D34Rr5	1
D34Rr6	1
D34Rr7	1/6
D34Rr8	1/6
D34Rr9	1/6
C26Rr2	1/2
C26Rr3	1/4
C26Rr4	1/4
C26Rr5	1/4
C26Rr6	1/4

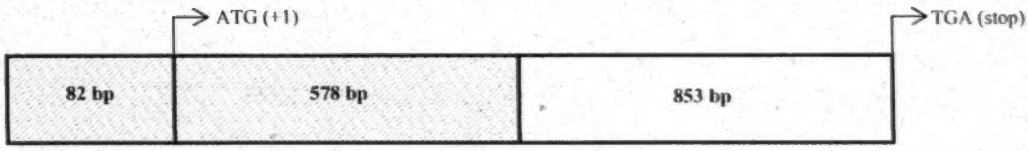


Figure 3-1 *: Coding region of the OST1 gene*

82 bp upstream of the start site and first 578 nucleotides of the *OST1* gene were sequenced using the plasmid primers YEp351-F and YEp351-R, which were intended to sequence *COX17*.

HUMAN COX17 'PSEUDOGENE'

Human copper transport is presumed to be analogous to that in yeast, with COX17, SCO1 and COX11 required for transport of copper to human cytochrome oxidase. During the characterization of the human *COX17* gene, a human *COX17* pseudogene (*COX17P*) was identified (Punter et al., 2000). Due to the genomic structure of the pseudogene and its apparent lack of expression in a lymphoblastoid cell line, it was labeled a non-functional, processed pseudogene. In the past, pseudogenes have been regarded as non-functional fragments of DNA resembling a known gene, however recent studies have begun to identify pseudogenes with important cellular functions. For example, the *Makorin1-p1* pseudogene, which contains the first 700 nucleotides of the *Makorin1* gene, was found to regulate and maintain a high level of expression of the *Makorin1* gene (Hirotsune et al., 2003). This finding and the fact that no mutations in *COX17* have yet been found to underlie human COX deficiency, support the possibility that COX17P might have a cellular function, either as an mRNA transcript or as a protein. I hypothesized that COX17P provides functional redundancy in the presence of *COX17* mutations. In order to support this hypothesis, COX17P would have to function similarly to COX17.

Genetic Ancestry

The *COX17* gene was first identified in *Saccharomyces cerevisiae* and proposed to encode a copper chaperone (Glerum et al., 1996a) that was later identified in humans (Amaravadi et al., 1997), pig (Takenouchi et al., 1999) and mice (Kako et al., 2000) and others. In humans, a second *COX17* locus was identified on chromosome 13q14-q21 by Punter et al. (2000) and proposed to be a processed pseudogene. A *COX17* pseudogene, however, is not present in the porcine and mice genomes. The 5'UTR of *COX17* and *COX17P* diverge 72 bp upstream of the methionine start site and 138 bp downstream of the TGA stop signal. Sequencing of the chimpanzee genome was recently completed by The Chimpanzee Sequencing and Analysis Consortium. I searched the chimpanzee genome for sequence similar to the human genomic *COX17* and *COX17P* sequence and identified the chimpanzee *COX17* sequence on chromosome 3 and the chimpanzee

COX17P sequence (*cCOX17P*) on chromosome 13. I found that *cCOX17P* was in a region (500 kb) of conserved synteny in humans. Only the human and chimpanzee genomes contain a *COX17* pseudogene, suggesting it retrotransposed into the genome recently in evolution. I have examined the chimpanzee *COX17* genomic sequence and found its gene organization to be identical to that of human *COX17*, as represented by Punter et al. (2000). Exon 1 contains 86 bps of 5' UTR and 107 bps of coding DNA followed by a 2 kb intron. Exon 2 contains 85 bps of coding DNA and 4 bps of 3' UTR. The TGA stop codon is located at position 190 within exon 2. The second intron is 5.5 kb, followed by the third exon, which contains 141 bps of 3' UTR. The chimpanzee pseudogene lacks introns and is thus predicted to be a processed pseudogene having the same genomic organization as the human pseudogene (Punter et al 2000). I was able to predict the coded protein sequence of chimpanzee COX17 and COX17P and align the human, chimpanzee, mouse, and yeast COX17 protein sequences (Figure 3-2). The human and chimpanzee COX17 amino acid sequences are identical; however, their pseudogenes each contain three amino acid changes. Both the human and chimpanzee pseudogene share the P11L amino acid change. In addition, cCOX17P contains a V5A and a L48V amino acid change, while human COX17P contains the K18R and A25T amino acid change (Figure 3-2).

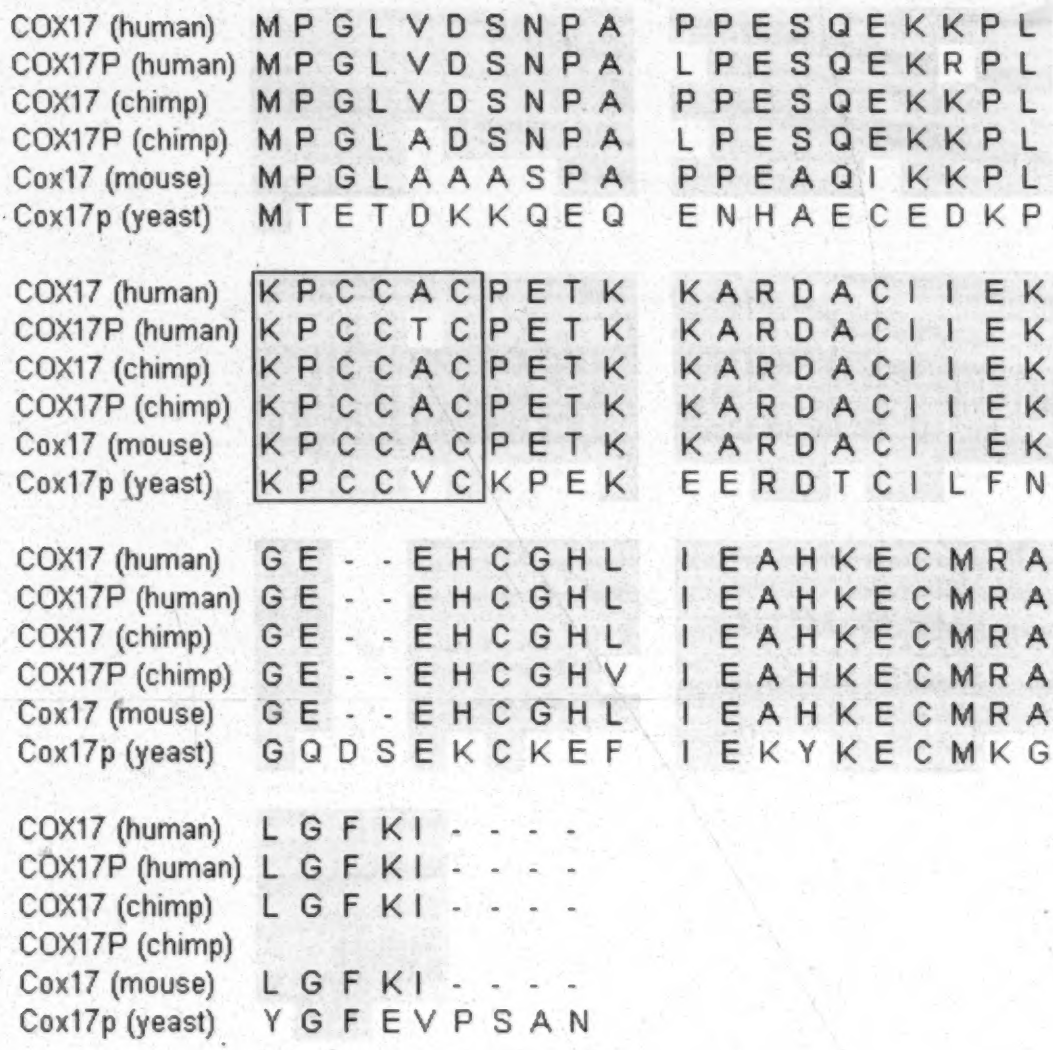

COX17 (human) M P G L V D S N P A P P E S Q E K K P L
COX17P (human) M P G L V D S N P A L P E S Q E K R P L
COX17 (chimp) M P G L V D S N P A P P E S Q E K K P L
COX17P (chimp) M P G L A D S N P A L P E S Q E K K P L
Cox17 (mouse) M P G L A A A S P A P P E A Q I K K P L
Cox17p (yeast) M T E T D K K Q E Q E N H A E C E D K P
COX17 (human) K P C C A C P E T K K A R D A C I I E K
COX17P (human) K P C C T C P E T K K A R D A C I I E K
COX17 (chimp) K P C C A C P E T K K A R D A C I I E K
COX17P (chimp) K P C C A C P E T K K A R D A C I I E K
Cox17 (mouse) K P C C A C P E T K K A R D A C I I E K
Cox17p (yeast) K P C C V C K P E K E E R D T C I L F N
COX17 (human) G E - - E H C G H L I E A H K E C M R A
COX17P (human) G E - - E H C G H L I E A H K E C M R A
COX17 (chimp) G E - - E H C G H L I E A H K E C M R A
COX17P (chimp) G E - - E H C G H V I E A H K E C M R A
Cox17 (mouse) G E - - E H C G H L I E A H K E C M R A
Cox17p (yeast) G Q D S E K C K E F I E K Y K E C M K G
COX17 (human) L G F K I - - - -
COX17P (human) L G F K I - - - -
COX17 (chimp) L G F K I - - - -
COX17P (chimp)
Cox17 (mouse) L G F K I - - - -
Cox17p (yeast) Y G F E V P S A N

Figure 3-2 : *COX17 Alignment*

An alignment of human, chimpanzee, mice and yeast COX17 proteins and of the proposed human and chimpanzee COX17P polypeptide sequences. The copper-binding domain, KPCCXC, is boxed.

Structural Prediction of Human COX17 and COX17P

COX17 is found in many species; however the yeast Cox17p structure is currently the only model available (Abajian et al., 2004). I submitted the human COX17 amino acid sequence to Swiss PDB (Guex and Peitsch, 1997), which predicted the human COX17 protein to be structurally similar to yeast Cox17p (Figure 3-3). Unfortunately, the first 14 amino acids were not included in the structural prediction, likely because the corresponding region in yeast Cox17p has no secondary structure. Like yeast Cox17p, human COX17 is predicted to have two alpha helices and an unstructured N-terminus. The cysteines (shown in yellow in Figure 3-3) located within the copper-binding domain form a 'cove' to facilitate copper binding in both the human and yeast COX17 proteins. Using Swiss PDB, I mutated the human COX17 residues K18R and A25T to resemble COX17P (Figure 3-4). Although A25T is in the copper-binding domain, the predicted COX17P structure maintains a copper-binding 'cove.' The K18R residue does not appear to alter the structure of COX17P, as it is located in an unstructured region of the N-terminus. Since there appears to be little structural change in the predicted copper-binding domain of COX17P, COX17P may be able to function similarly to COX17.

Figure 3-3 : *Predicted Human COX17 structure*

The Cox17p NMR structure from yeast has been determined and is shown on the left. The predicted human COX17 structure, shown on the right, is similar to yeast Cox17p. The yellow residues represent the cysteines located in the copper-binding domain and the pink sphere represents copper bound by yeast Cox17p, as determined by its NMR structure. The protein structures were modified using Swiss PDB viewer (version 3.7) (Guex and Peitsch, 1997).

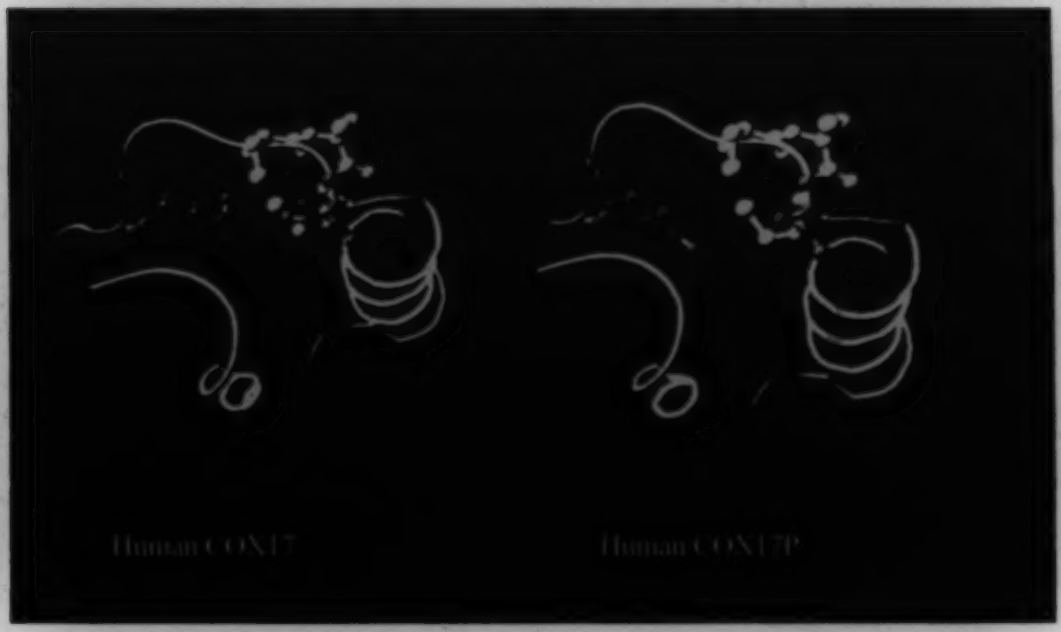


Figure 3-4 : *Predicted structural differences between human COX17 and COX17P*
The predicted human COX17 and COX17P structures both contain two alpha helices and an unstructured N-terminus. The yellow residues represent the cysteines located in the copper-binding domain and the orange residues represent the residues that differ in COX17P and COX17. The protein structures were modified using Swiss PDB Viewer (version 3.7) (Guex and Peitsch, 1997).

Functional complementation

COX17 proteins localize to mitochondria, likely for copper delivery to SCO1. The three amino acid changes in COX17P that may not affect mitochondrial localization or copper binding, but may affect copper delivery to SCO1. For COX17P to have a functionally redundant role in the COX assembly pathway, COX17P must be able to function similarly to COX17. The ability of COX17P to complement a yeast *cox17* knockout would suggest that COX17P functions similarly to yeast Cox17p in delivering copper to Scolp.

A yeast *cox17* knockout strain was transformed with high-copy plasmids containing human *COX17* and *COX17P*. The transforming plasmid, pMGL4-hCOX17, is a yeast expression vector containing human *COX17* and pMGL4-hCOX17P is a yeast expression vector containing human *COX17P*. When present in multicopy, human *COX17* was able to rescue the respiration-deficient phenotype of the yeast Δ*cox17* strain, with a growth rate only slightly slower than that of wild-type yeast (Figure 3-5). When high-copy *COX17P* is transformed into the yeast *cox17* null mutant, respiratory competency is gained to the same extent as *COX17*. The *cox17* null mutant strain transformed with empty pMGL4 vector did not show any growth on EG medium (Figure 3-5). As a positive control, yeast *cox17* null mutants were transformed with a high-copy plasmid containing yeast *COX17*. As expected, yeast COX17 could rescue the respiratory deficiency (Figure 3-5). This result demonstrates that despite the three amino acid changes in COX17P, it can maintain a function similar to COX17.

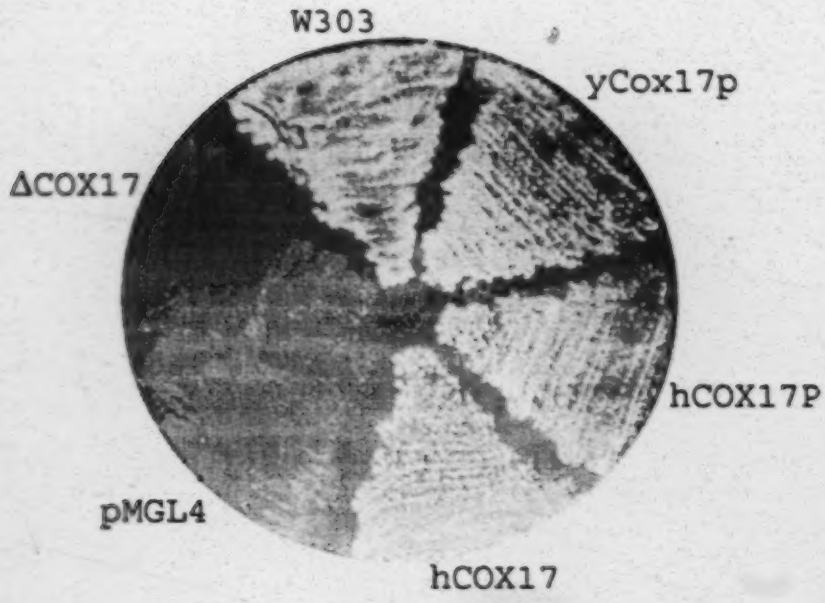


Figure 3-5 : *Functional complementation of yeast cox17 null mutant*

Yeast transformants, along with the wild-type parent strain (W303) and the *cox17* null mutant (ΔCOX17), were streaked onto YPD (rich glucose) plates and grown at 30°C for two days. Following replica plating, strains were scored for growth after 40 hours on the EG plate and photographed. hCOX17 = *cox17* null mutant transformed with a plasmid expressing human *COX17* (pMGL4-hCOX17); pMGL4 = *cox17* null strain transformed with empty plasmid; hCOX17P = *cox17* null mutant transformed with a plasmid expressing *COX17P* (pMGL4-hCOX17P); yCox17p = *cox17* null mutant transformed with a plasmid expressing yeast *COX17* (pG74/ST21)

Cellular Localization of COX17 and COX17P

Previous investigations of mouse Cox17 demonstrated localization to the perinuclear region rather than to mitochondria, which suggested that mammalian COX17 proteins played a role in synthesis or secretion of neuropeptide hormones (Kako et al., 2000). However, human COX17 has since been localized to the cytoplasm and mitochondria for copper delivery to SCO1 and subsequently to COX (Punter, 2003). COX17 and COX17P only differ by three amino acids, one of which is located in the copper-binding domain. Depending on the functional importance of these amino acids, localization or copper-binding could be altered. According to the predicted COX17P structure, copper binding is not likely to be affected. To determine if the three amino acids altered in COX17P affect mitochondrial localization, I examined COX17P by subcellular fractionation and immunofluorescence.

Both C-terminal and N-terminal FLAG-tagged *COX17* and *COX17P* constructs were created in the mammalian expression vector, pcDNA3.1, for protein expression and further protein isolation by subcellular fractionation. Punter et al. (2003) could only detect N-terminal FLAG-tagged COX17 proteins by Western blot and not C-terminal FLAG-tagged COX17 proteins. As with COX17, I could only detect the N-terminal FLAG-tagged COX17P protein by Western blot. Since there are only three amino acid differences between COX17 and COX17P, we are unable to create an antibody that can distinguish between COX17 and COX17P. Therefore, COS-7 cells were transfected with the N-terminal FLAG-tagged *COX17* (*FLAG-COX17*) or *COX17P* (*FLAG-COX17P*) constructs and grown for three days to allow for adequate protein expression. Three days post-transfection, the cytosolic and mitochondrial fractions were isolated and analyzed by Western blot using α-FLAG antibody (Figure 3-6). FLAG-COX17P and FLAG-COX17 signals were detected in both the mitochondrial and cytosolic fractions at 21.5 kDa, which was determined by comparison to a known molecular weight ladder. There was no detectable FLAG-COX17 and FLAG-COX17P in mitochondrial and cytosolic fractions of untransfected cells. To verify the specificity and purity of the subcellular fractionation, the two fractions were probed with antibody against exclusively mitochondrial or cytosolic marker proteins. Human COX I, the largest subunit of COX, is an integral

component of the mitochondrial inner membrane, and thus serves as a distinct mitochondrial marker. Western blotting with a monoclonal antibody to COX I confirmed the presence of this protein in the mitochondrial fractions from both the transfected and untransfected cells, but not in either of the post-mitochondrial supernatant fractions (Figure 3-6). This indicated that the presence of detectable FLAG-COX17 and FLAG-COX17P in the cytosolic fraction is not due to a failure to pellet broken or damaged mitochondria. Human farnesyltransferase is a cytosolic protein involved in farnesylation of a variety of intracellular protein targets. Immunoblotting of the mitochondrial and cytosolic COS-7 fractions with a monoclonal antibody (IB7) to the α subunit of the human farnesyltransferase was detected in both of the post-mitochondrial supernatant fractions, thus demonstrating the lack of cytosolic contaminants in the mitochondrial fraction (Figure 3-6).

To confirm the cellular localization of FLAG-COX17 and FLAG-COX17P as determined by subcellular fractionation, I assessed untransfected COS-7 cells and COS-7 cells transfected with FLAG-tagged *COX17* and FLAG-tagged *COX17P* by immunofluorescence (Figure 3-7). COS-7 cells transfected with N-terminal FLAG-tagged *COX17* and *COX17P* constructs were immunostained with α-FLAG antibody, demonstrating detectable FLAG-COX17 and FLAG-COX17P in structures surrounding the nucleus. The COS-7 cells were also incubated with the mitochondrial-specific dye, MitoTracker Red CMX-Ros. When the immunofluorescence images from the α-FLAG antibody and MitoTracker Red were merged, the same structures detected by the α-FLAG antibody were stained by MitoTracker Red, indicating that FLAG-COX17 and FLAG-COX17P colocalize with mitochondria. This confirms the localization of COX17 and COX17P to the mitochondria, as observed in the subcellular fractions examined by Western blots. To confirm the specificity of the α-FLAG antibody, untransfected COS-7 cells were incubated with α-FLAG antibody and MitoTracker Red. In this case, the MitoTracker Red staining did not overlap with the green fluorescent signal from FITC-conjugated secondary antibody (Figure 3-7). To verify the colocalization of mitochondrial protein with MitoTracker Red, untransfected COS-7 cells were also stained with anti-cytochrome *c* antibody and the mitochondrial-specific dye. Both

MitoTracker Red and antibody against cytochrome *c*, a mitochondrial intermembrane space protein, detected the same cellular structures (Figure 3-7).

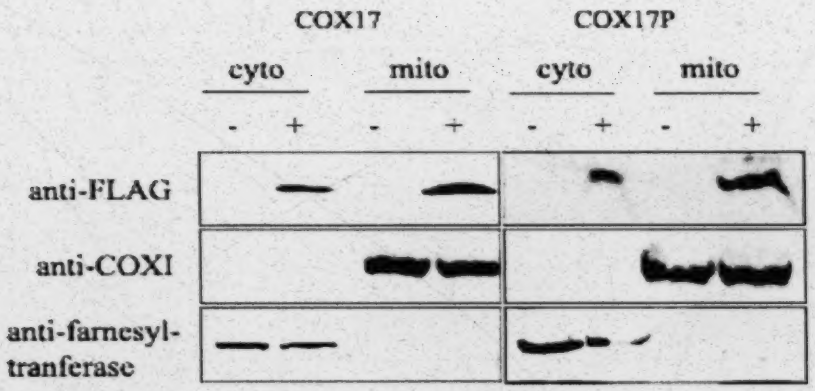


Figure 3-6 : *Subcellular Fractionation of transfected and untransfected COS-7 cells* COS-7 cells were transfected with pCDNA3.1-hCOX17NFL or pCDNA3.1-hCOX17NFLP, which contain the human *COX17* gene and *COX17P* with a FLAG epitope fused in-frame at the 5' end, respectively. Postmitochondrial supernatant (40 μg) and mitochondrial fractions (40 μg) from transfected and untransfected COS-7 cells were separated on 12% polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies against human COXI (a mitochondrial protein) and human farnesyltransferase (a cytosolic protein). Blotting with the α-FLAG antibody was carried out in a similar manner except that 60 μg of total cellular protein was loaded in each lane. - = untransfected COS-7 cells, + = transfected COS-7 cells, cyto = postmitochondrial supernatant fraction, mito = mitochondrial fraction

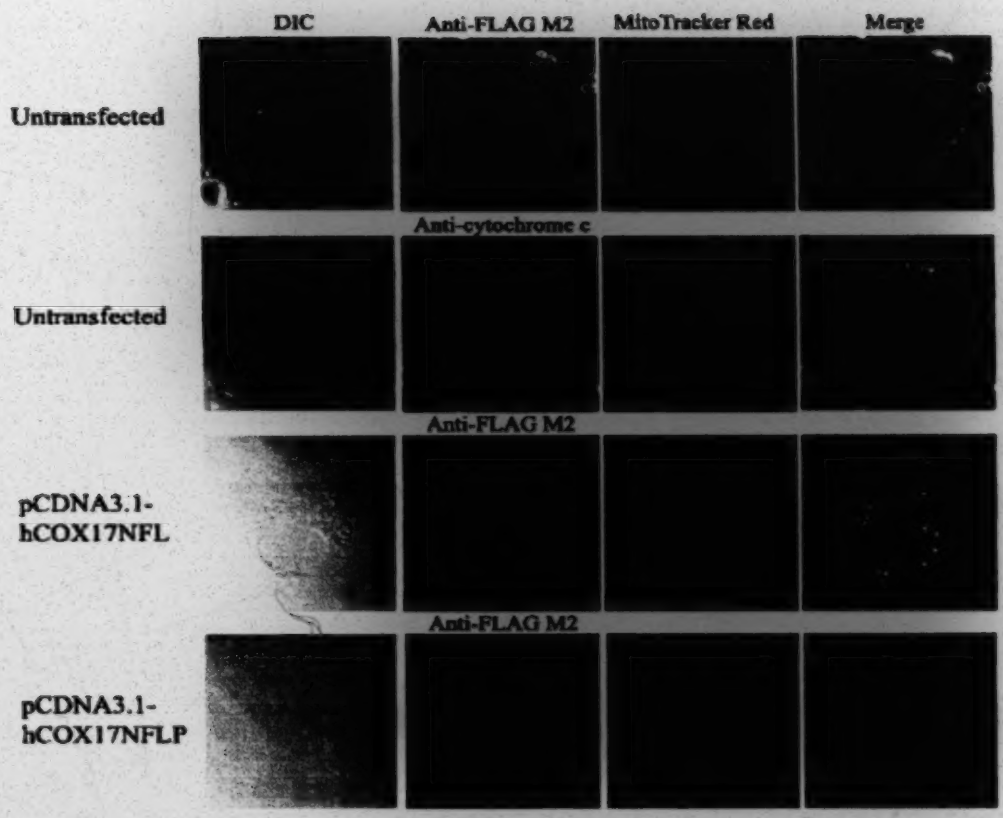


Figure 3-7 : *Immunofluorescence of transfected and untransfected COS-7 cells*
COS-7 cells transiently transfected with pCDNA3.1-hCOX17NFL and pCDNA3.1-hCOX17NFLP were incubated with α-FLAG antibody (green) and MitoTracker Red (red). The yellow represents the merging of the red and green signals and indicates colocalization of tagged COX17 with mitochondria. As a negative control for the α-FLAG antibody, untransfected COS-7 cells were incubated with α-FLAG antibody (green) and MitoTracker Red (red). Untransfected COS-7 cells were also incubated with MitoTracker Red and antibody against cytochrome *c*, a known mitochondrial intermembrane space protein. DIC = differential interference contrast

Expression of human COX17 and COX17P

To initiate gene expression, some genes contain upstream promoter or enhancer sequences recognized by transcription factors. Many genes have TATA boxes or transcription factor binding sites for transcription initiation. The yeast nuclear-encoded COX subunits and the mouse *COX17* gene have been found to contain upstream transcription binding sites recognized by the transcription factors NRF-1, NRF-2 and Sp1 (Ongwijitwat and Wong-Riley, 2005; Grossman and Lomax, 1997). I made several attempts to identify transcription factor binding sites upstream of *COX17P*, however no obvious sites were found. I used TATA-box prediction software to analyze 1 kb of upstream *COX17P* sequence and identified several potential TATA boxes at 200 bp, 380bp, 425 bp, 475 bp, 720 bp and 800 bp upstream of the putative *COX17P* start site (Milanesi et al., 1996). During transcription, most messages are polyadenylated for transcript stability prior to translation. Human *COX17* has a known polyA signal sequence and a post-transcription polyA tail (Punter et al., 2000). *COX17P* does contain a polyA signal, but it is unknown whether the *COX17P* transcript becomes polyadenylated. I have not identified any *COX17P* ESTs in the NCBI database (as of December 2005), which may suggest that *COX17P* is not polyadenylated, since EST databases use an Oligo-dT primer for first-strand synthesis.

To determine whether or not *COX17P* is expressed, I obtained total cellular RNA from liver, spleen, kidney, lung, thymus, heart, brain and retina tissues and performed reverse transcription (RT) reactions using an Oligo-dT primer for first-strand synthesis of the cDNA. The *COX17* cDNA was subsequently amplified using *COX17*-specific primers, demonstrating ubiquitous expression of *COX17* (Figure 3-8), which confirms previous Northern blot results from Punter et al. (2000). *COX17P* was not amplified from any of the tissues, suggesting either a lack of expression or the inability to produce *COX17P* cDNA using an Oligo-dT primer because the *COX17P* transcript is not polyadenylated. The RT reactions were repeated using a reverse primer specific to *COX17P* and *COX17* (HCOX17HP-R) for first-strand synthesis of cDNA. Under these experimental conditions, *COX17P* was successfully amplified by PCR from spleen, liver and thymus cDNA samples, using a *COX17P*-specific forward primer (HCOX17P-F) and

the reverse primer (HCOX17HP-R) used for the RT reaction. To ensure my primers were specific to *COX17P*, I cloned the genomic *COX17P* PCR product into the pGEM T-Easy vector and verified that the sequence of the amplified product was indeed *COX17P* (data not shown). *COX17P* is a processed pseudogene, therefore, no primers could be selected for RT-PCR that would specifically amplify cDNA rather than genomic DNA. Although no signals were present in the negative control or in the water control, we wanted to confirm RNA samples were not contaminated with genomic DNA and giving rise to the *COX17P* product. I attempted to amplify *PAX6*, an unrelated gene, from the liver, spleen and thymus cDNA samples and while the expected 300 bp product was obtained with genomic DNA, *PAX6* was not amplified in the liver, spleen or thymus samples. Thus, genomic DNA was not contaminating the RNA samples and accounting for the amplification of *COX17P*.

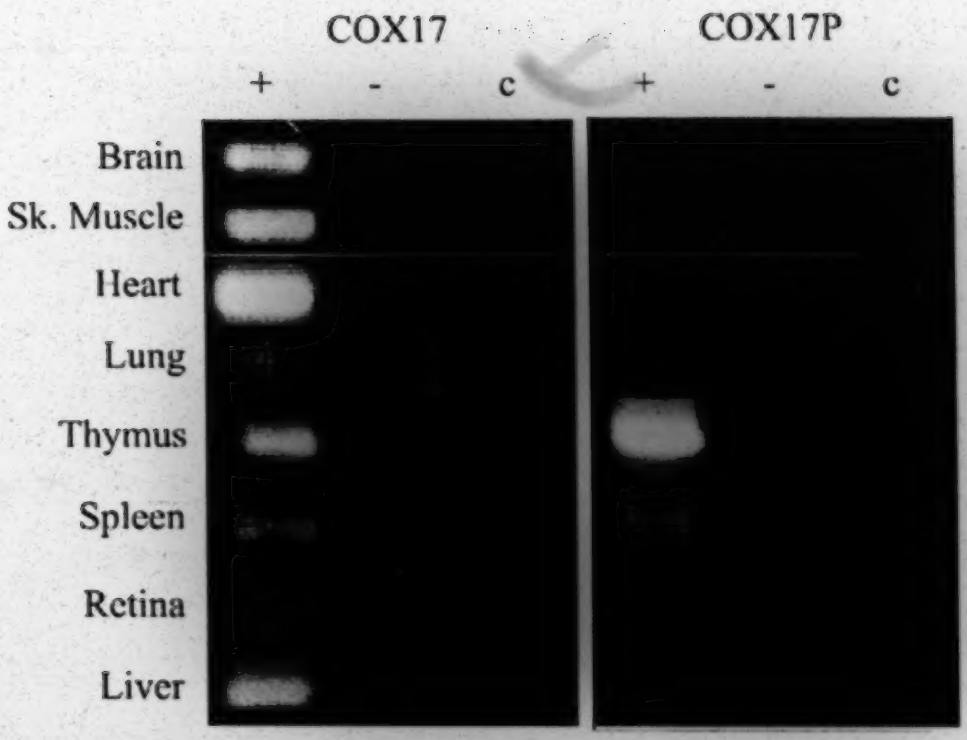


Figure 3-8 : *Expression panel from human tissues*

Reverse transcription was performed on total cellular RNA isolated from human brain, skeletal muscle, heart, lung, thymus, spleen, retina and liver, using an Oligo-dT primer for first-strand synthesis of *COX17* or HCOX17HP-R for first strand synthesis of *COX17P*. *COX17* was found to be ubiquitously expressed and *COX17P* was found to be expressed in the thymus, liver and spleen. + = reverse transcriptase added for first strand synthesis; - = no reverse transcriptase added for first strand synthesis; c = water control, no RNA added, reverse transcriptase was added.

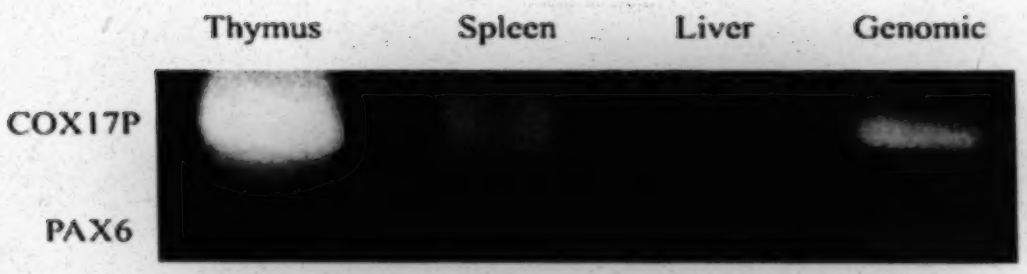


Figure 3-9 : *PAX6 control for Thymus, Spleen and Liver samples*

To ensure that *COX17P* was not amplified from contaminating genomic DNA in the RNA samples, the liver, spleen and thymus cDNA samples were subjected to PCR with primers specific for genomic *PAX6*, an unrelated gene. The lack of a *PAX6* PCR product demonstrates that *COX17P* was amplified from cDNA and not from genomic DNA contaminants.

The thymus and spleen play an integral role in the immune system by producing cells important for protecting the body against foreign material and helping to remove foreign materials from the blood. The thymus is the site of T-cell generation and maturation, which protects the body from infection, whereas the spleen primarily functions to trap antigens and filter the blood of antigens and lymphocytes (Goldsby, 2003). The spleen is also important in mounting immune responses to antigens in the blood stream. Tissue-specific expression of *COX17P* in the liver, spleen and thymus may suggest a specific role for *COX17P* in the immune system. I obtained several immune cell lines to further investigate *COX17P* expression. I isolated total RNA from transformed B-cells (221 and Raji), T-cells (Jurkat and RPMI 8866), natural killer cells (YTS and NK92), monocytes (THP-1), cytotoxic lymphocytes (hCD8), hepatoma cells (HUH7 and HepG2) and HeLa cells and performed RT reactions to create *COX17* and *COX17P* cDNA. *COX17P* was amplified using *COX17P*-specific primers in all cell lines, except cytotoxic lymphocytes, HeLa cells and both of the hepatoma cell lines (Figure 3-10). *COX17* was ubiquitously expressed in all cell types examined. To ensure that no genomic DNA contaminated the cDNA samples, I attempted to amplify *PAX6* by PCR from each cDNA sample (Figure 3-11). *PAX6* was amplified in genomic DNA, but was not amplified from any of the cDNA samples, indicating that *COX17P* was amplified from cDNA and not contaminating genomic DNA.

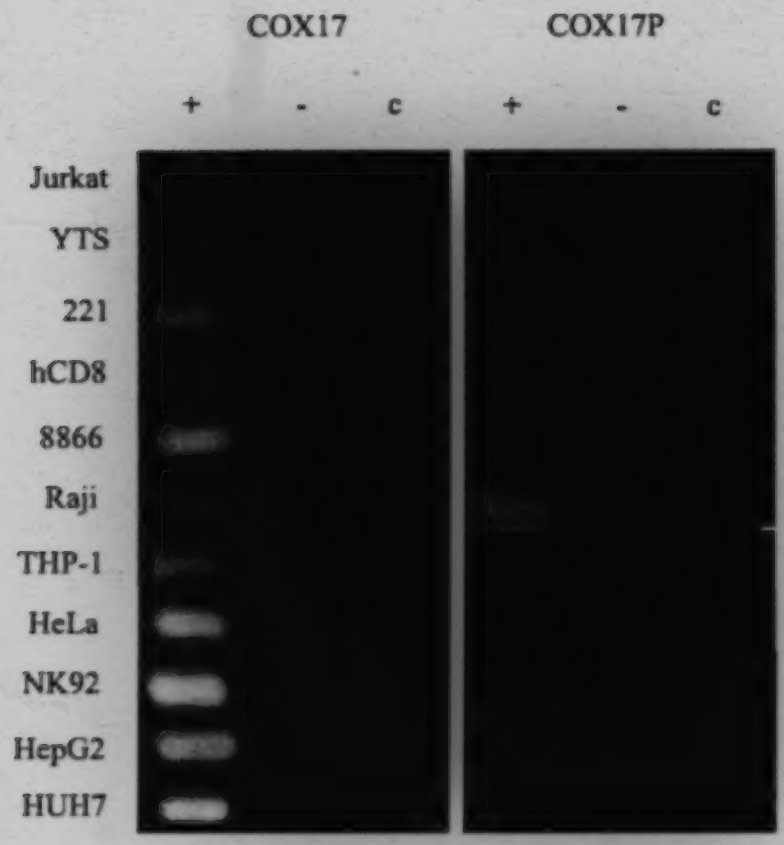


Figure 3-10 : *Expression of COX17 and COX17P in various immune cell lines*
Reverse transcription was performed on total RNA isolated from Jurkat, YTS, 221, hCD8, 8866, Raji, THP-1, HeLa, NK92, HepG2 and HUH7 cell lines, using an Oligo-dT primer for first strand synthesis of *COX17* or HCOX17HP-R for first strand synthesis of *COX17P*. *COX17* was ubiquitously expressed in all cell lines and *COX17P* was expressed in the Jurkat, YTS, 221, 8866, Raji, THP-1 and NK92 cell lines.

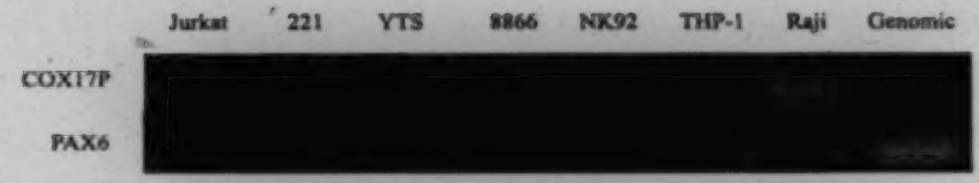


Figure 3-11 : *PAX6 control for immune cell lines*

To ensure that *COX17P* was not amplified from genomic DNA contamination in the cDNA samples, each sample was subjected to PCR with primers specific for genomic *PAX6*, an unrelated gene. The lack of a *PAX6* PCR product demonstrates that *COX17P* was amplified from cDNA and not from genomic DNA contaminants.

Chapter 4 ❖ Discussion

MITOCHONDRIAL COPPER TRANSPORT STUDIES IN YEAST

Several independent investigations of Sco1p, Cox17p and Cox11p have formed the current model of copper transport to the mitochondrial enzyme, cytochrome oxidase. Sco1p was first identified in 1988 as a COX assembly factor important for COX II accumulation (Schulze and Rodel, 1988) and was later suggested to contain the copper-binding domain, CxxxC (Glerum et al., 1996b). Cox17p was first identified as a potential copper-binding protein (Glerum et al., 1996a), which was found to be suppressed by high-copy Sco1p (Glerum et al., 1996b). These findings and that there is loss of Cox2p in *cox17* mutants suggested that the Cu_A site in COX acquired copper through a relay system involving Cox17p and Sco1p (Dickinson et al., 2000)(Horng et al., 2004) (Figure 1-6). Cox17p was localized to both the cytosol and mitochondria (Beers et al., 1997), which suggested Cox17p chaperoned copper from the cytosol to the IMS for copper delivery to Sco1p and Cox11p. Another SCO protein, Sco2p, was found to suppress *cox17* point mutants; however, its role in the cell is still unclear.

The initial model of copper transport to COX suggested that Cox17p chaperoned copper from the cytoplasm to the mitochondrial IMS. However, recent experimental findings suggest that Cox17p is transported across the OMM in an unfolded *apo-* form (Meseke et al., 2005). As well, Δ*cox17* strains containing Cox17p fused to Sco2p in the IMM are respiratory competent (Maxfield et al., 2004), which, in fact, means that Cox17p may not chaperone copper from the cytosol to the IMS, as the model proposes. Therefore, Cox17p must bind copper after import into the IMS, but then two questions arise: 1) What is the source of the copper? and 2) Why would Cox17p deliver copper to Sco1p, when Sco1p could obtain copper directly? There are several possible ways by which Cox17p could receive copper. One source could be the free pool of copper in the mitochondrial matrix (Cobine et al., 2004), however for Cox17p to be accessible to the matrix copper, copper would need to be chaperoned into the IMS. It may also be possible that the IMS contains protein-free copper or that another IMS copper-binding protein provides copper to Cox17p, for example, Cox23p. Δcox23 strains are rescued with high-

copy Cox17p in the presence of copper, which suggests Cox23p functions in a common pathway with Cox17p acting downstream of Cox23p (Barros et al., 2004). Cox23p, not implicated in the disulphide relay system may, in fact, chaperone copper to the IMS to provide Cox17p with copper. Cox19p was considered as alternate copper chaperone, however, it imports into the IMS by the disulfide relay system like Cox17p (Chacinska et al., 2004), presumably unfolded and unbound to copper. The second question posed addresses why Cox17p would be imported into the IMS without copper and then become copper loaded for delivery to Sco1p, as it would be more efficient for Sco1p to receive copper directly. In addition to copper delivery to the Cu_A site, Sco1p has been proposed to function as a mitochondrial copper-dependent redox signaling molecule (Williams et al., 2005). The redox switch of Sco1p may require interaction with Cox17p, which may be necessary to prepare Cox2p for copper acceptance. Cox17p and Sco1p have been indirectly shown to interact, which may be required for 'activation' of the redox-switch. Thus, Cox17p may have a secondary cellular function; 'activation' of redox-signaling and copper transport. Although the currently accepted model clearly describes a mechanism of copper transport to COX, it is becoming apparent that other proteins, such as Cox23p, are, in fact, involved in copper transport or that the proteins currently thought to be involved have a secondary function, such as Sco1p and possibly Cox17p.

Sco1p acts a high-copy suppressor of cox17 mutants, whereas Sco2p acts as an allele-specific high-copy suppressor of cox17 mutants

To further characterize the proteins currently thought to be involved in copper transport, I examined the ability of Sco1p and Sco2p to suppress a null *cox17* strain and several *cox17* point mutants. The *cox17* mutants I investigated can be broken into four groups: *cox17* knockout (Δ*cox17*); *cox17* copper-binding mutants (C24R, C26R); *cox17* import mutants (incapable of importing into mitochondria – C47R, C57Y); other *cox17* point mutants (importance of these residues unknown – D34R, D34V, R33D). The results of the multicopy suppression screen demonstrated suppression of the *cox17* mutants, including Δ*cox17* by high levels of Sco1p. This suggested that whatever the function of

Sco1p, it was not entirely dependent on Cox17p, since respiration was restored in the absence of Cox17p. Respiration is also restored in *cox17* copper-binding mutants and *cox17* import mutants, which further supports the hypothesis that Sco1p can bind copper independently from Cox17p. As well, the ability of Sco1p to suppress Δ*cox17* suggests that a secondary copper transport pathway exists, but functions less efficiently. If Cox23p is an IMS copper chaperone, maybe it can partially replace the function of Cox17p by providing copper to Sco1p in the absence of Cox17p. Sco1p could also be receiving copper from a cytosolic copper chaperone or a mitochondrial matrix copper chaperone that provides the IMS with copper. The suggestion that an interaction between Cox17p and Sco1p exists and facilitates function is supported by the fact that respiration rates in strains containing mutant *cox17* are more comparable to wild-type rates than Δ*cox17* strains. Interestingly, the site of the *cox17* mutation was not important for restored respiration, suggesting that no particular Cox17p residue is necessary for Sco1p and Cox17p interaction. However, this study examined only eight Cox17p residues, and thus does not eliminate the possibility that other Cox17p residues are important for interaction with Sco1p. If a particular residue were important, I would not expect respiration to be restored following transformation with high-copy Sco1p or Sco2p.

I also performed a multicopy suppressor screen with high-copy Sco2p, which demonstrated allele-specific suppression of *cox17* mutants. Sco1p and Sco2p have been shown to form heteromeric complexes (Lode et al., 2002) and both are thought to bind copper, but are not capable of complementing one another (Glerum et al., 1996b). In contrast to Δ*sco1* mutants, Δ*sco2* mutants are respiratory competent, suggesting Sco2p is not essential for oxidative growth. Sco2p has been shown to interact with Cox2p, independent from copper and Sco1p, suggesting that Sco2p can also deliver copper to the Cu_A site (Lode et al., 2002). Copper delivery by Sco2p is insufficient for oxidative growth and is not able to substitute for Sco1p activity. If a function of Sco1p and Sco2p overlaps, then the dominant copper pathway seems to involve Sco1p and not Sco2p. Respiration in a Δ*cox17* strain was not restored by high levels of Sco2p, suggesting that Sco2p plays another function in addition to copper delivery to Cox2p. But, as stated, Sco2p is unable to replace Sco1p's activity and thus must have a secondary function that

is yet to be defined. Since Sco2p, like Sco1p, has sequence similarities to peroxiredoxins, it may function in mitochondrial redox signaling (Williams et al., 2005). The results of this study also demonstrate that overexpression of Sco2p could compensate for the *cox17* mutants, C24R, C47R, C57Y, D34R and D34V. Sco2p suppression is independent from Cox17p copper binding and import, since suppression of copper-binding, import and "unknown" mutants was observed. High-copy suppression of C57Y is supported by Glerum et al. (1996b) who found Sco2p to suppress the C129/U1 mutant, which contained a C57Y *cox17* mutation. Sco2p could not suppress C26R, a copper-binding mutant, and R33D, an "unknown" mutant, which may suggest an important interaction with Sco2p and these Cox17p residues. An interaction between Sco2p and Cox17p may be necessary for Sco2p to interact with Cox2p during copper delivery, since C26 is located in the copper-binding domain. To further investigate a possible interaction between Sco2p and Cox17p, it may be useful to create double *cox17* mutants, with one of the two mutations being at the C26 or R33 residue. In this case, we would not expect Sco2p to suppress a respiratory deficiency. Further biochemical analyses, such as immunoprecipitation, would also be required to confirm this hypothesis.

Identification and characterization of revertants

The results of the Sco1p suppression study strongly suggest that other proteins are involved in copper transport to the IMS or to COX. In order to identify other gene products involved in copper transport, I undertook a multicopy suppressor screen of several *cox17* point mutants, using a previously created yeast genomic library (L13). Typically, multicopy suppressors are thought to act downstream of the gene they suppress. For example, the suppression of Δ*cox17* by Sco1p suggests that Sco1p acts downstream of Cox17p in the copper transport pathway (Glerum et al., 1996a). The L13 library was transformed into the C26R and D34R *cox17* mutants. Ideally, the number of transformants should be triple the number of genes in the genome in order to ensure complete coverage during a single genetic screen. The yeast genome contains approximately 6,000 genes, therefore 18,000 transformants from a single transformation

experiment would be ideal. The maximum number of transformants I could obtain after three independent transformations was 6,300. Along with a low number of transformants, there appeared to be several colonies that did not segregate with the correct auxotrophies and had gained respiratory competency on EG. Since the W303 yeast strain is auxotrophic for histidine, leucine, tryptophan, uracil and adenine and the *cox17* mutant strains were disrupted with *TRP1*, contained a *URA3* marker on the plasmid with the mutant *cox17* alleles and were transformed with a leucine L13 library plasmid, we would have expected the transformants to require histidine and adenine for growth on minimal media. Yeast strains grown under selective pressure can begin to revert. Thus, to tease out whether the revertants were a result of selective pressure, or a result of the transformation process, I plated D34R and C26R at high-density on EG. I observed colonies that gained respiratory competency, but lost prototrophic selection for either the *URA3* marker plasmid containing the mutant *cox17* allele or the *TRP1* locus at the *cox17* knockout site. Because these mutant strains were reverting on EG plates, it is unlikely that the transformation process caused the revertants previously observed. I chose to examine the D34R and C26R colonies that required histidine, leucine and adenine for growth and that had gained respiratory function. Despite growth on EG, these revertants contained the *URA3* plasmid with the mutant *cox17* allele and the *TRP1* locus at the *cox17* knockout site. Since I wanted to use these revertants to generate a yeast genomic library, I screened for mutations in the known suppressors *SCO1* and *SCO2*, as well as in the *cox17* mutant plasmid. However, no mutations were identified. Interestingly, when I amplified *cox17* by yeast colony PCR in five of the D34R revertants (r1-5) and sequenced the product, I identified a portion of the *OST1* gene. The *URA3* plasmid primers that amplified *OST1* did not match any sequence in the yeast genome, and thus were specific to the *URA3* plasmid. The mutant *cox17* sequence was amplified using *cox17*-specific primers, thus was still present in the genome of these revertants. The *cox17* plasmid may have integrated into the yeast genome, explaining why, despite several attempts, the *URA3* plasmid could not be isolated and why I have sequenced a portion of the *OST1* gene. Altogether, the revertants had gained respiratory competency, likely due to an extragenic suppressor in a gene other than *SCO1* and *SCO2*.

Now that these revertant strains have been screened for mutations in the *SCO1* and *SCO2* genes, as well as reversion of the mutant *cox17* allele, it will be useful to create a genomic library for a multicopy suppressor screen in *cox17* mutants in order to identify the site of the reversion. Following transformation and selection of colonies that have gained respiratory competency, the genomic library plasmid can be isolated to determine the gene sequence that leads to respiratory gain. I created a yeast *ΔSCO1/ΔSCO2/ ΔCOX17* strain that will be useful to generate a genomic library for a multicopy suppressor screen in *cox17* and *sco1* mutants. These genomic libraries will be useful to identify novel gene products involved in the copper transport pathway to COX or in a parallel pathway.

Summary

COX biogenesis is a complex processes that requires numerous assembly factors to coordinate the assembly of mtDNA- and nDNA-encoded subunits within the IMM and the delivery and placement of the prosthetic groups. The results described in this section of the thesis clearly suggest that other gene products are involved in copper delivery to COX and that our current model of copper transport may need to be modified. Cox17p can not act as a mitochondrial copper chaperone, since import occurs in an unfolded state and unbound to copper (Meseke et al., 2005). Another copper protein, possibly Cox23p, if it is not imported by the disulfide relay system, may chaperone copper from the cytosol to the IMS for delivery to Cox17p (Barros et al., 2004). Cox17p could also receive copper from a matrix chaperone protein that provides the IMS with copper (Cobine et al., 2004). Once Cox17p is bound to copper, it may function in a heteromeric complex with Sco1p or Sco2p or simply form transient interactions required for function. The interaction of Sco1p with Cox17p may facilitate mitochondrial redox signaling and/or copper delivery to Cox2p. A Sco2p/Cox17p complex may function to deliver copper to Cox2p, however less efficiently than the mechanism of copper delivery by Sco1p. A third possibility is that Sco1p interacts with, or receives copper from, another copper protein, as in the case of a *Δcox17* strain. Through a multicopy suppressor screen we may be able

to identify suppressors of *cox17*, or *sco1* mutants and thus further define mitochondrial copper transport. Identifying all of the factors involved in copper delivery to COX will advance our understanding of the role of metallochaperones in COX assembly, as well as in human COX deficiency.

HUMAN COX17 'PSEUDOGENE'

The biogenesis of cytochrome oxidase is a complex process that involves the coordination and placement of ten nDNA-encoded subunits and three mtDNA-encoded subunits within the IMM. As well, many COX assembly factors are essential to complete holoenzyme assembly. Mutations have been identified in all three of the mtDNA-encoded subunits, *COX I-III* and in the nDNA-encoded COX assembly factor genes *SCO1*, *SCO2*, *COX10*, *COX15*, *LRPPRC* and *SURF1* in human COX deficiencies. Unfortunately, as we do not yet completely understand the process of COX assembly, the underlying genetic cause for many COX deficient patients is unknown. Due to the vast number of candidate genes, the identification of mutations leading to COX deficiency has been difficult. Some of the assembly factors implicated in COX deficiency are involved in copper and heme A placement within the holoenzyme. However, not all candidate genes involved in these pathways, such as *COX17* and *COX11* (both of which are essential for respiration), have yet been found to contain mutations in human COX deficiency.

The *COX17* gene has been identified in the yeast, mouse, rat, cow, pig, chimpanzee and human genome. A second *COX17* locus is also present in the human (Punter and Glerum, 2003) and chimpanzee genome. Due to a lack of exon/intron organization and a lack of expression in a lymphoblastoid cell line, the second human *COX17* locus was proposed to be a processed pseudogene (Punter et al., 2000). The mouse genome does not contain a *COX17* pseudogene and homozygous *Cox17* knockout mice are embryonic lethal (Takahashi et al., 2002). Since Δ*Cox17* mice are embryonic lethal, then you may assume that a *COX17* null human would also lead to embryonic lethality. This could explain why no human *COX17* mutations have yet been found (Horvath et al., 2005; Darin et al., 2003; Horvath et al., 2000; Sacconi et al., 2003). However, humans contain a

second *COX17* locus, *COX17P*, which could provide a COX17 protein and prevent embryonic lethality.

Processed pseudogenes are non-functional sequences of genomic DNA that derived from a functional gene. These sequences are thought to be transcriptionally and translationally silent due to premature stop codons or frameshift mutations, which *COX17P* lacks. However, several non-functional and functional pseudogene transcripts have been identified in humans (Balakirev and Ayala, 2003) and some have been found to regulate the gene from which they derived. For example, the *Makorin1-p1* pseudogene, which contains the first 700 nucleotides of the *Makorin1* gene, was found to regulate and maintain a high level of expression of the *Makorin1* gene (Hirotsune et al., 2003). I proposed that human *COX17P*, if it were to be expressed and transcribed, would lead to functional redundancy in the COX assembly pathway. For COX17P to be functionally redundant, however, COX17P must function similarly to human COX17.

COX17P localizes to mitochondria and may bind copper

Both human and yeast COX17 proteins are localized to both the cytoplasm and mitochondria for copper delivery to SCO1 (Beers et al., 1997; Punter, 2003). Like human COX17, COX17P also localizes to both the cytosol and mitochondria, as demonstrated by subcellular fractionation and immunofluorescence. This contradicts Kako et al. (2000), who proposed that mammalian Cox17 functions in the secretion of neuropeptides, since Cox17 localized to the perinuclear region and not to mitochondria. The proposed COX17P protein sequence contains three amino acid substitutions (P11L, K18R, A25T), which do not appear to affect mitochondrial localization or copper transport to COX. This is not surprising, as the corresponding amino acid residues P11, K18 and A25 are not utilized in the yeast disulfide relay system for mitochondrial import (Chacinska et al., 2004). As well, copper binding and delivery to SCO1 does not appear to be hindered by the A25T amino acid change in the copper-binding domain, since COX17 and COX17P could complement the yeast *cox17* knockout strain at near wild-type levels. Not surprisingly, the A25 residue is not conserved and likely can withstand an amino acid

change, as in COX17P (Punter and Glerum, 2003). If COX17P were to be expressed and transcribed, it could function similarly to COX17, by binding and delivering copper to SCO1. Examination of the predicted COX17P protein structure demonstrated little difference in the copper-binding 'cove' when compared to Cox17p (Abajian et al., 2004), which also suggests that COX17P could bind copper. Unfortunately, since the amino acid sequences of COX17 and COX17P differ only by three, we are unable to create specific antibodies against COX17 and COX17P. Protein analysis of COX17P would help determine whether or not the *COX17P* transcript is translated. Altogether, a COX17P protein could import into the mitochondria and may bind copper, just as COX17 does, despite the presence of three amino acid changes. These results support the hypothesis that COX17P could provide functional redundancy in the presence of a *COX17* mutation.

Expression profile demonstrates COX17P likely does not prevent development of human COX deficiency

To date, no human *COX17* mutations have been identified in patients with COX deficiencies. *COX17* is a candidate gene for human COX deficiencies due to its importance in COX assembly and its role in copper delivery to SCO1. Both yeast *sco1* and *cox17* knockout strains are COX deficient (Glerum et al., 1996a; Beers et al., 1997). Thus, we may expect mutations in human *COX17* and *SCO1* to lead to human COX deficiencies. Homozygous Δ*Cox17* mice are embryonic lethal (Takahashi et al., 2002), which may explain why no *COX17* mutations have been identified in human COX deficient patients, although embryonic lethality has not been observed in humans. Human *SCO1* mutations lead to COX deficiencies and in some cases, hepatopathy (Valnot et al., 2000a). Human *SCO2* mutations lead to fatal infantile cardioencephalomyopathy with COX deficiencies (Papadopoulou et al., 1999). Typically, muscle, heart and brain tissues are most affected by COX deficiency. Thus, let's assume human *COX17* mutations lead to COX deficiency in muscle, heart and brain tissues. In order for COX17P to provide functionally redundant copies of COX17 proteins and prevent the development of human COX deficiency, we would expect *COX17P* to be expressed in muscle, heart and brain

tissues. However, *COX17P* was found to be expressed in the liver, spleen and thymus and not expressed in tissues typically affected by COX deficiency. If *COX17* mutations lead to COX deficiencies in the brain, muscle and heart tissues, then *COX17P* would not be able to provide functionally redundant copies of COX17 proteins.

COX17P expression was initially examined in lymphoblastoid cells by reverse transcription of total cellular RNA using an Oligo-dT primer for first strand synthesis (Punter et al., 2000). Since the majority of expressed messages are polyadenylated during transcription, Oligo-dT primers can be used to create cDNA (Buratowski et al., 2005). However, *COX17P* could only be reverse transcribed with a *COX17P*-specific primer and not with an Oligo-dT primer, suggesting that *COX17P* may not polyadenylated, despite having a polyA signal. This may explain why *COX17P* expression was not identified in the lymphoblastoid cell line and why no *COX17P* ESTs have been identified, since EST databases are created using Oligo-dT. Non-polyadenylated transcripts are typically unstable and readily degraded in the cell, which decreases the chance that a message is translated (Buratowski et al., 2005). Since *COX17P* transcripts may not be polyadenylated, they may be easily degraded, thus reducing the likelihood of *COX17P* translation. Since *COX17P* cDNA could not be created using an Oligo-dT primer, we do not know the size of the transcript. If *COX17P* is located within a transcript much larger than the approximated 500 bp, the transcript may also have a decreased chance of being translated into a protein.

Expression of COX17P is unique to immune cell lines

I have demonstrated that *COX17P* is expressed in the spleen, liver and thymus. The thymus is the site of T-cell generation and maturation, and the spleen filters blood of antigens and lymphocytes (Goldsby, 2003). Since both the thymus and spleen play an important role in immunity, I investigated *COX17P* expression in cells of the immune system. There are two main lineages of immune cells: 1) lymphoid and 2) myeloid. The lymphoid cell lineage generates B-cells, T-cells and natural killer cells and the myeloid cell lineage generates monocytes, neutrophils and others (Goldsby, 2003). Interestingly,

COX17P is expressed in B-cells, T-cells, natural killer cells and monocytes, suggesting that COX17P does not play a specific role in either the lymphoid or myeloid cell lineages. Identifying *COX17P* expression in several immune cells may suggest a role for COX17P in immunity. However, since *COX17* is ubiquitously expressed in immune cells, COX17P would not be required to provide COX17 proteins to these cells. Human COX deficiency has not yet been associated with the immune system, and thus COX17P does not likely provide functional redundancy in the COX assembly pathway in immune cells.

COX17 has been a suggested target for non-small cell lung cancer (NSCLC) as *COX17* transcripts are overexpressed in lung cancer cell lines, which leads to an increase in COX activity (Suzuki et al., 2003). If *COX17* transcripts could be downregulated in lung cancer cells, then a decrease in COX activity could reduce the level of tumorigenic proliferation. Since *COX17* was overexpressed in cancer cells, maybe COX17P or COX17 leads to a decrease in cell death. Since there is a high rate of cell death in immune cells following activation, *COX17* and *COX17P* expression may lead to an overabundance of *COX17* transcripts and thus may enhance tumorigenic proliferation. The analysis of caspase-3 cleavage (an apoptotic indicator) following granzyme-B treatment (an apoptotic inducing agent) in *COX17*- and *COX17P*-transfected HeLa cells, could determine whether or not COX17 and COX17P play a role in apoptosis. This method and fluorescence activated cell sorting (FACS) are standard techniques for identify and characterizing the apoptotic pathway and could explain a possible function for COX17P and thus explain *COX17P* expression in immune cells.

Unfortunately, we do not know the size of the *COX17P* mRNA transcript or know what promoter sequence initiates transcription. Interestingly, a gene, *LCP1*, is located upstream of *COX17P* that exhibits a similar expression profile to COX17P. *LCP1* is not expressed in HeLa cells but was found to be expressed in the liver and in a variety of human tumor cell lines, including T-cells, natural killer cells, monocytes and B-lymphoblasts, which is identical to the expression profile of *COX17P*. All of the immune cell lines I investigated were transformed cells of hemopoietic origin, thus it may be

possible that *COX17P* is expressed under the control of the *LCP1* promoter and explain the immune-specific expression profile that *COX17P* exhibited.

Although *COX17P* transcripts have been identified in the liver, spleen, thymus and in several immune cell lines, we have no evidence that *COX17P* transcripts are translated into a protein. Unfortunately, due to high sequence similarity, antibodies can not be created to distinguish between COX17 and COX17P. To understand whether or not COX17 and COX17P play distinct functional roles, we could perform RNA interference. By creating a short, transcript-specific sequence for transfection into *COX17P*-expressing cells, we would knockdown gene expression and observe the phenotypic effect. Isolation of total cellular RNA and analysis of post-knockdown expression levels by quantitative PCR would assess whether COX17P expression is being knocked down and following phenotypic observation we could further our understanding of the function of COX17P.

Summary

The experiments outlined in this section of the thesis demonstrate that COX17P could function similarly to COX17 in any tissue it were expressed and translated. *In vitro* protein analysis demonstrated that COX17P, like COX17, could localize to mitochondria and functionally complement a yeast *cox17* knockout, suggesting that, functionally, COX17P could replace COX17. Because no *COX17* mutations have been identified in human COX deficiency, I sought to determine whether COX17P could replace COX17 and thus prevent the development of a COX deficiency. Although COX17P appears to function similarly to COX17, it is not expressed in tissues typically affected by human COX deficiency, such as skeletal muscle, heart and brain. As well, these results do not demonstrate whether COX17P is, in fact, translated into a protein, which would affect its ability to be functionally redundant. COX17P expression in immune cell lines may suggest a role for COX17P in immunity, however that is only a postulation and is yet to be defined. Further analysis of COX17P as a protein involved in cell death may explain a role for COX17P in immune cell turnover.

To maintain COX function in the cell, numerous assembly factors are required to coordinate the expression, translation and localization of nuclear and mitochondrial-encoded subunits in the IMM. A subset of these assembly factors are necessary for the delivery and placement of the prosthetic groups within COX. Failure to complete COX assembly leads to respiratory deficiency in yeast. The associated human disorders present with lactic acidosis, encephalopathy, myopathy and liver and kidney problems, as well as such complex and common neurodegenerative diseases as Alzheimer disease (Cottrell et al., 2002). These diseases are known to have an underlying mitochondrial pathology, frequently associated with problems in COX function. Many COX deficient patients have been found to bear mutations in *SCO1*, *SCO2*, *SURF1*, *COX10*, *COX15* and *LRPPRC*. However, several investigators have examined COX deficient patients for mutations in the candidate genes, *COX17*, *COX19* and *COX11*, but none have been identified. The assembly of COX in the IMM, and its functional role in energy production, is of significant importance in the study of neurodegenerative disease. However, little is known about the mechanisms and pathways that mediate COX assembly and function, particularly the delivery and placement of the active site molecules within COX. Continued studies of the molecular pathway of copper delivery to COX is integral in the understanding, treatment and prevention of neurodegenerative diseases that present with or stem from COX deficiencies.

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